Physiological Zoology

APRIL 1952

Oxygen Consumption in Microscopically Active and Resistant Encysted Cells
Joseph Bell Hutner, Elsie Hoag Lee, and William L. Irwin 1

Osmotic Activity and Physical State of Intracellular Glycogen
J. Gordon Kaplan 14

Oxygen Consumption in Cultures Fresh-Water Fishes
Alfred E. Chidd and David W. Fisher 27

Effect of Temperature on Trout as Assayed by Injection of Staphylococcus
F. E. Rogers and L. E. Foster 37

Influence of Dietary Deficiency on Hair Development and Pigmentation
Harold Rusch 48

Growth and Differentiation in the Adult Tubercle-bearing Bass
Percy Jones 69

Histochemistry of Larval Skin in *Rana pipiens*
Percy T. Kelley and Kate C. Kelley 79

Changes during the Endocystic Development of the Japanese Beetle
Earl Hamilton 93

Growth Curves of Interspecific and Intraspecific Competition in Daphnia
Clarence Ray Carpenter and Mary Pyecroft Pennak 115

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PHYSIOLOGICAL ZOÖLOGY

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SUCCINIC DEHYDROGENASE IN MITOTICALLY ACTIVE AND BLOCKED EMBRYONIC CELLS[1]

JOSEPH HALL BODINE, KIAO-HUNG LU, AND WILLIAM L. WEST
Zoölogical Laboratory, State University of Iowa, Iowa City, Iowa

THAT succinic dehydrogenase is widely distributed in organisms seems well established (Lardy, 1949). The degree of activity of this enzyme has been shown to vary for different tissues and even for the same tissue under varied physiological conditions. Most succinoxidase (succinic dehydrogenase plus cytochrome oxidase) is thought to be located for the most part in vertebrates in the large granule fraction of the cytoplasm—the mitochondria; and in the case of rat liver some 70–80 per cent of the activity of the original homogenate has been found in these structures (Hogeboom, Schneider, and Pallade, 1947). The importance of the cytochrome system and the part played by it in the normal reactions of succinic dehydrogenase have been repeatedly pointed out (Lardy, 1949). Preliminary results of experiments with the colorimetric triphenyltetrazolium chloride method for estimating succinic dehydrogenase with the embryo of the grasshopper failed to show the presence of the enzyme in this organism (Bodine and Fitzgerald, 1949*b*). Further investigations, however, using manometric as well as colorimetric techniques, have shown a rather unique and striking activity of the succinic dehydrogenase during periods of active growth and cell division, as well as for the mitotically inactive or blocked stages in the development of the embryo. The present paper is concerned with results of experiments dealing with the changes in the succinoxidase system of mitotically active and blocked embryonic cells of the grasshopper, *Melanoplus differentialis*.

[1] Aided by a grant from the National Institutes of Health. Technical assistance of Etta Andrews is gratefully acknowledged.

MATERIAL AND METHODS

Embryos were dissected free of yolk from eggs of this grasshopper. Details as to methods were similar to those already pointed out in previous publications (Bodine and Lu, 1950). Both mitotically active (pre- and postdiapause) as well as blocked (diapause) embryos have been employed. The chronological as well as morphological ages of embryos were carefully controlled, since this point has been found of paramount importance in investigations of enzyme activity. Ringer's solution, phosphate-buffered, pH 6.8, as well as sucrose (0.25 M) was used as a suspension medium. Differences in reactions of homogenates in Ringer and sucrose are of interest when compared with similar data for vertebrate tissues. These points will be discussed below. Homoge-

nates of embryos were prepared with motor-driven glass pestles and fractional centrifugations, and separations were carried out as previously indicated (Bodine and Lu, 1950). All reagents used were C.P., and concentrations indicated are final ones in respiration flasks. Oxygen consumption was measured at 25° C. with standard Warburg manometers and respiration flasks of 5-ml. capacity. Microdifferential manometers were employed where amounts of oxygen were too small for significant determinations by the Warburg apparatus. One hundred embryos per milliliter of medium were used in all experiments, and in those cases where intracellular constituents were employed these, too, are from 100 embryos/ml of medium. Further details of methods are discussed below in dealing with the results of the experiments.

RINGER'S SOLUTION AND SUCROSE (0.25 M) AS SUSPENSION MEDIA FOR INTACT EMBRYOS, HOMOGENATES, AND INTRACELLULAR CONSTITUENTS

Embryos, dissected from eggs and freed of yolk in Ringer's solution, were divided into two equal lots. One lot was washed several times in Ringer and then left suspended in this medium. The other lot was washed with 0.25 M sucrose and finally left suspended in it. The pH of both solutions was 6.8. The endogenous oxygen uptake of the two lots was simultaneously followed for periods of 2 or more hours at 25° C. The results of typical experiments are graphically shown in Figure 1. An inspection of this figure shows no significant differences in the oxygen uptake for the intact embryos in the two media. Similar results are obtained for both mitotically active and blocked embryos.

Homogenates prepared from intact embryos suspended in both Ringer or sucrose showed rather marked differences both in physical and in physiological properties. In sucrose, homogenates appear more viscous or sticky than those in Ringer, and, when centrifuged, the precipitate (nuclei) is found much clumped together in the sucrose and quite free in the Ringer. When nuclei are homogenized in sucrose, the nuclear homogenate becomes very sticky and extremely difficult, if not impossible, to handle with

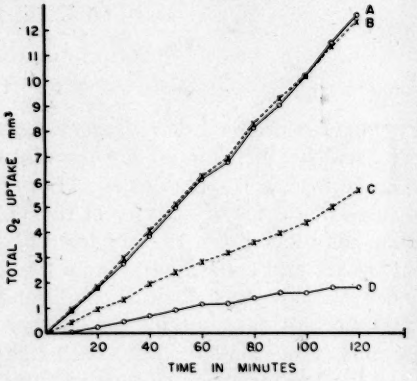

FIG. 1.—Shows the effect of Ringer and 0.25 M sucrose on the endogenous oxygen uptake of intact diapause embryos and their homogenates. *A* = intact embryos in Ringer; *B* = intact embryos in sucrose; *C* = homogenates in Ringer; *D* = homogenates in 0.25 M sucrose.

pipettes. Endogenous oxygen uptake of embryo homogenates prepared in Ringer is at a markedly higher level than for corresponding ones prepared in sucrose. This point is graphically shown in Figure 1. Nuclear homogenates from both active and blocked embryos behave similarly (Fig. 2).

When homogenates from embryos are fractionally centrifuged into a supernatant (cytoplasmic) and a nuclear layer, the marked differences in endogenous oxygen uptake of homogenates in Ringer and sucrose can be partially explained. Nuclei in sucrose, as indicated above, become sticky and clumped. In this condition the oxygen uptake is

greatly reduced over that found for intact nonclumped nuclei in Ringer (Fig. 2). This lowered oxygen uptake of such nuclei would account for much of the decrease in oxygen uptake for the embryo

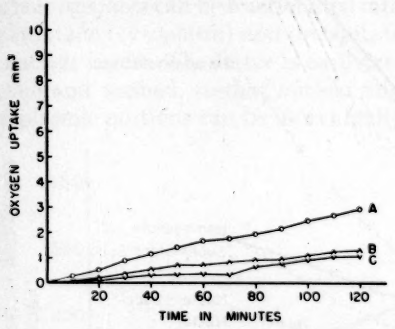

FIG. 2.—Shows the endogenous oxygen uptake of isolated intact nuclei in *A*, Ringer; *B*, nuclei from sucrose homogenates; *C*, nuclei suspended in sucrose immediately after isolation in Ringer.

homogenate in sucrose. As a matter of fact, the oxygen uptake of supernatants (cytoplasm minus nuclei) in sucrose and in Ringer is practically identical (Fig. 3). Succinate added to supernatants in sucrose show much greater stimulation of oxygen uptake than for those in Ringer (Fig. 3).

RESULTS

1. ACTION OF SODIUM SUCCINATE ON OXYGEN UPTAKE OF INTACT EMBRYOS, HOMOGENATES, AND INTRACELLULAR CONSTITUENTS

Sodium succinate when added to both diapause (blocked) and postdiapause (active) embryo homogenates produces stimulation of oxygen uptake, indicating the presence of a succinoxidase system. The relative degree of stimulation in the different physiological states of the embryo, however, indicates quite marked differences in the amounts or activities of the enzyme systems at these respective physiological levels of cellular activities. The endogenous oxygen uptake of the intact embryo seems little, if at all, affected by the addition of succinate, and this is probably associated with a marked degree of impermeability to the reagent. The homogenates as well as intracellular constituents, on the other hand, are

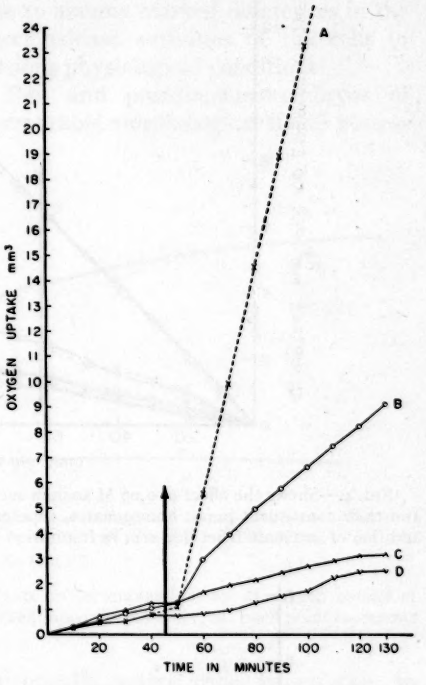

FIG. 3.—Shows effect of 0.18 M succinate upon the oxygen uptake of nuclei-free supernatant in 0.25 M sucrose and Ringer. Arrow indicates time that contents of side arms were introduced into respiration flasks. *A* = supernatant in sucrose, succinate added from side arm; *B* = supernatant in Ringer, succinate added from side arm; *C* = supernatant in Ringer, Ringer added from side arm; *D* = supernatant in sucrose, sucrose added from side arm.

markedly stimulated by the reagent. Typical results are graphically shown in Figures 4 and 5.

An examination of Figure 4 shows that the oxygen uptake of the intact embryo is not significantly, if at all, stimulated by additions of 0.06 M sodium succinate. Similar results have been found for all intact embryos, irrespective of their physiological states.

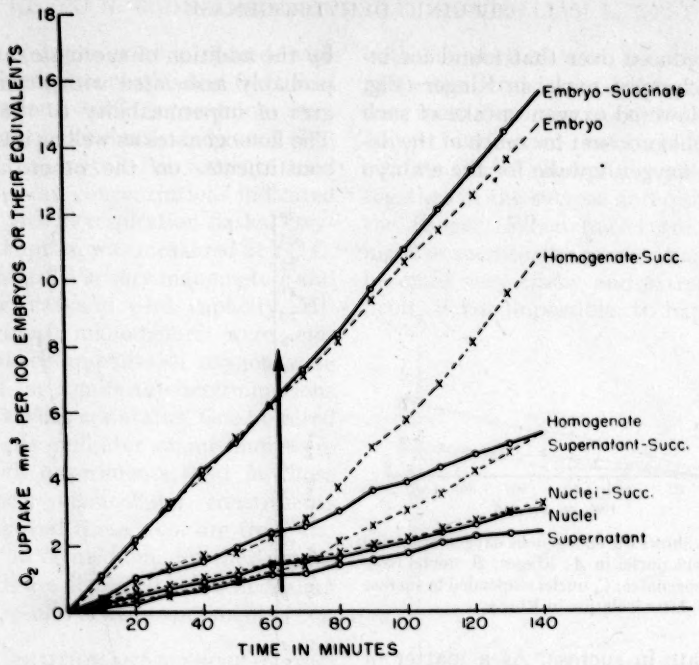

FIG. 4.—Shows the effect of 0.06 M sodium succinate on the oxygen uptake of intact diapause embryos and their constituent parts: homogenates, supernatant (cytoplasm), and nuclei (intact). Arrow indicates addition of succinate from side arm to respiration flask.

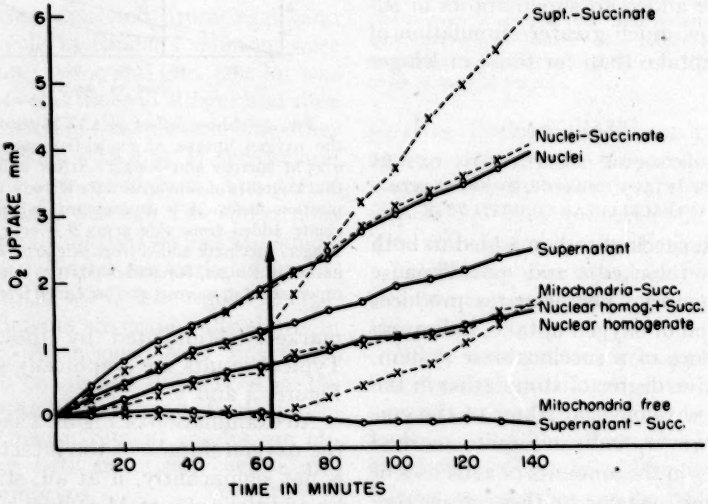

FIG. 5.—Shows the effect of 0.06 M sodium succinate on the oxygen uptake of cell (diapause) constituents. Otherwise similar to Figure 4.

The oxygen consumption of homogenates of embryos, however, is markedly stimulated by the addition of succinate, suggesting the presence of a succinoxidase system (Fig. 4). When centrifuged, the homogenates can be fractionated into supernatant (cytoplasm) and precipitate or nuclear layers. The latter is easily removed and washed, so that nuclear and cytoplasmic portions can be individually treated with succinate. The supernatant or cytoplasmic layer containing the large granules (mitochondria) is markedly stimulated by the addition of succinate, while the intact nuclei are apparently not at all affected (Fig. 5). Nuclear homogenates are also not stimulated by succinate (Fig. 5). Mitochondria, obtained by fractional centrifugation of the cytoplasmic supernatant, are strikingly stimulated by the addition of succinate, while the mitochondria-free supernatant gives no such response (Fig. 5). Such a reaction resembles that reported for mammalian cells by Hogeboom and others (Lardy, 1949; Hogeboom, Schneider, and Pallade, 1947) and would lead one to assume that the mitochondria contain the greater part, if not all, of the succinoxidase system of the embryonic cell. In general, similar responses to succinates are characteristic of all cells examined, whether in the mitotically active or blocked state. However, the extent of the response leads one to assume marked differences in the succinoxidase activities of the cells in various physiological conditions.

Pre- and postdiapause embryos of comparable morphological stages possess mitotically active cells, which can, in turn, be compared with similar cells in mitotically inactive or blocked states from diapause embryos. It is thus possible to compare the succinoxidase system of cells from embryos of relatively equal sizes but of different physiological states. Diapause or mitotically blocked embryos give homogenates which, upon addition of succinates, show marked increases in oxygen uptake, much in excess of that given by comparable homogenates made from postdiapause or mitotically active ones. With increasing concentrations of succinate, increased stimulation up to approximately 300 per cent for diapause homogenates and 140–50 per cent for postdiapause ones occurs (Fig. 6). One may reasonably infer, there-

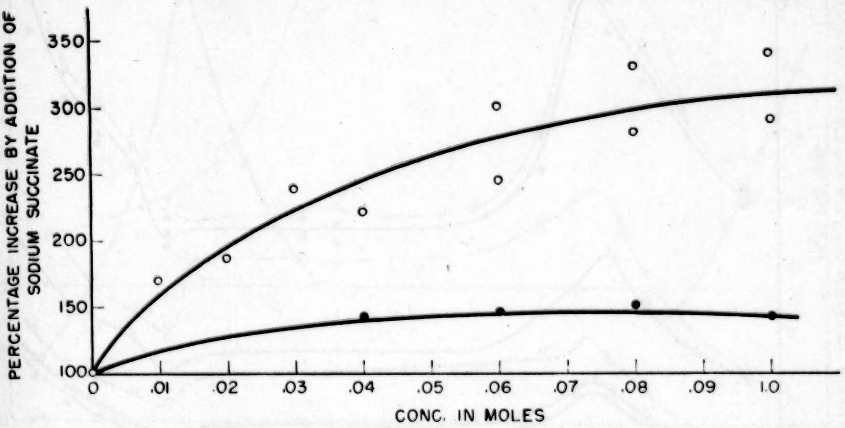

FIG. 6.—Shows effect of concentration of sodium succinate on percentage increase of oxygen uptake of homogenates from mitotically active (solid circles) and blocked (open circles) embryos. Each point represents averages from a minimum of sixteen determinations.

fore, that more active succinoxidase must be available in the blocked than in the active stages. Not only does this hold true for the homogenates, but the mitochondria, when fractionally centrifuged, show similar responses. In Figure 7 are diagrammatically shown the relative oxygen consumption rates for different physiological states of the intact embryo, homogenate, nuclei, and mitochondria when subjected to 0.06 M sodium succinate.

If one assumes the normal oxygen uptake of the homogenate of the blocked or mitotically inactive embryo to be 100 per cent and then expresses the rates of endogenous oxygen uptake of prediapause and postdiapause homogenates correspondingly, rather striking correlations between these and succinoxidase activities become apparent. Such relations are graphically shown in Figure 8. Inspection of this figure shows the endogenous oxygen uptake of the prediapause, developing homogenate to increase from the fifteenth day to approximately the nineteenth day at 25° C. With the onset of diapause or developmental block, the oxygen uptake falls to the diapause level, where it remains practically constant throughout the blocked or diapause period. With the cessation of diapause, the

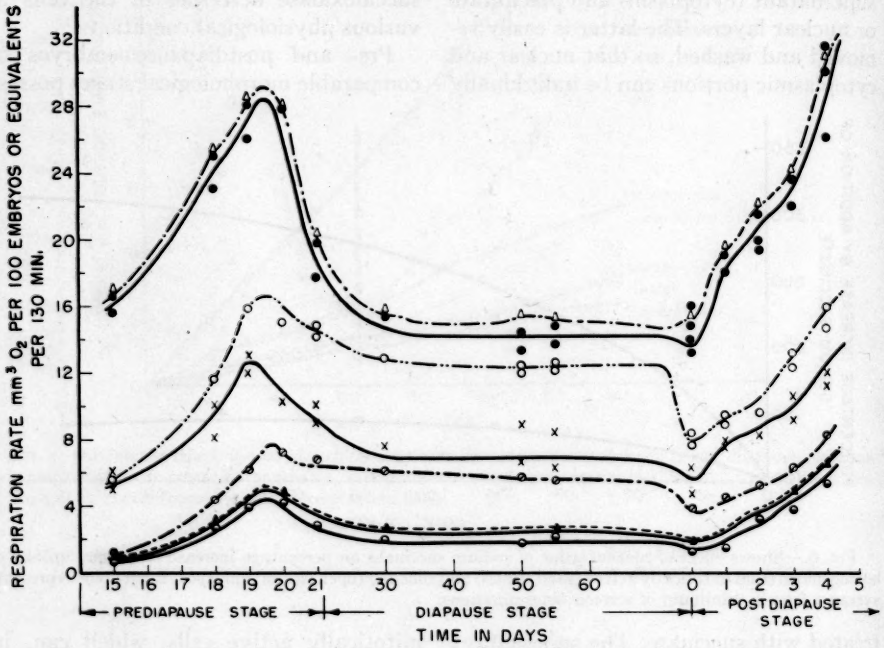

FIG. 7.—Shows effect of 0.06 M sodium succinate on the oxygen uptake of intact embryos, homogenates from embryos, supernatants (cytoplasm), and nuclei at different periods throughout embryonic development. Two top lines for intact embryos (solid circles and solid line for succinate; broken line and open circles for Ringer solution). Next two lines for homogenates (solid line and *X* for Ringer solution; broken line with open circles for succinate). Next two lines for supernatants (solid line for Ringer; broken line with open circles for succinate). Lowest lines for nuclei (solid line and open circles for Ringer; dotted broken line for succinate). Most marked differences in response to succinate are found in homogenate and supernatants (containing mitochondria). Nuclei do not react with succinate when thoroughly washed.

oxygen consumption increases markedly during postdiapause up to the time of hatching. When the increase of oxygen uptake, due to the addition of succinate, is similarly plotted, it can be seen that succinoxidase activity is low during early prediapause and gradually increases, reaching its maximum during the blocked or diapause period. During diapause, enzyme activity remains high and fairly constant, but, with the breaking of the block and the beginning of active development, succinoxidase activity drops to prediapause levels. Prediapause embryos of 18–19 days are morphologically similar to diapause and to 2–3-day postdiapause ones, so that differences in enzyme activity of embryos of these stages cannot be due primarily to differences in size or bulk of the embryo. It has previously been noted that during diapause a cyanide-insensitive respiration seems to predominate, while during both pre- and postdiapause a cyanide-sensitive respiration is most prominent (Bodine and Boell, 1934). Further points relative to the significance of these changes in succinoxidase activity will be pointed out below.

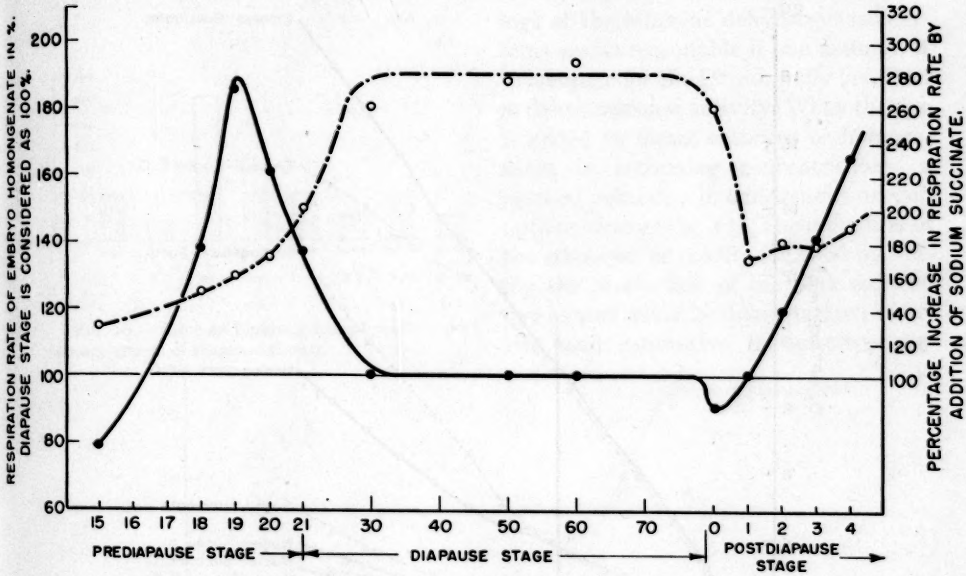
RESPIRATION RATE OF EMBRYO HOMOGENATE IN %. DIAPAUSE STAGE IS CONSIDERED AS 100%.
PERCENTAGE INCREASE IN RESPIRATION RATE BY ADDITION OF SODIUM SUCCINATE.
PREDIAPAUSE STAGE
DIAPAUSE STAGE
POSTDIAPAUSE STAGE

FIG. 8.—Shows relative effects of 0.06 M sodium succinate upon percentage oxygen uptake of homogenates throughout embryonic development (ordinate at right). Normal endogenous respiration of diapause homogenates taken as 100 per cent and respiration throughout development expressed in corresponding percentages (ordinate at left). Open circles, succinate stimulation; solid circles, endogenous oxygen uptake.

2. EFFECTS OF SODIUM SUCCINATE AND ETHYL CARBAMATES

Ethyl carbamate (urethane) has been shown markedly to lower reversibly the oxygen uptake of the grasshopper embryo and other biological forms (Bodine and Fitzgerald, 1949*a*). The manner of action of this drug, although not well understood, has been thought by some to be largely upon the dehydrogenase systems of the living cell (Lardy, 1949). When applied to the normal and succinate-stimu-

lated homogenates of the grasshopper embryo, a marked reduction in the endogenous as well as in the stimulated fractions of the oxygen consumption is noted. Figures 9 and 10 show graphically the results of typical experiments with these

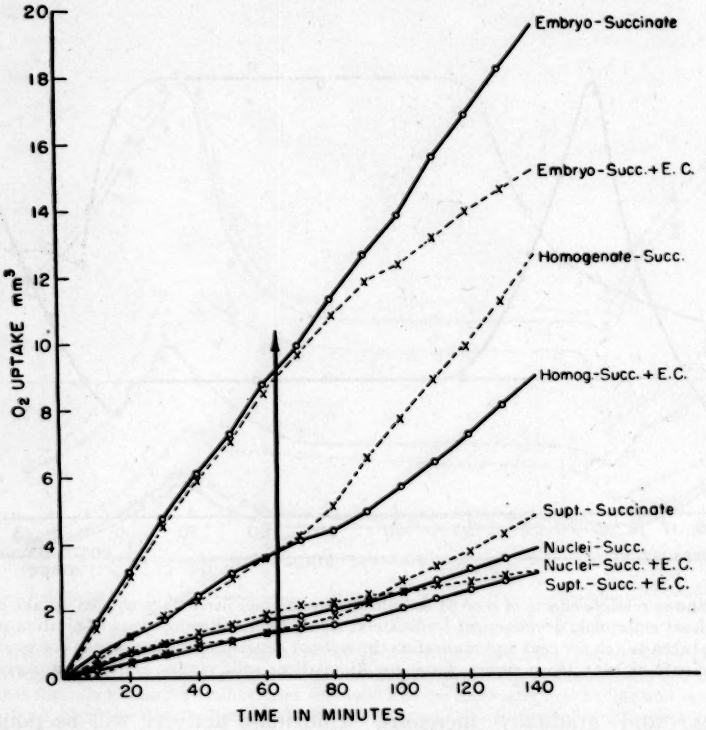

FIG. 9.—Shows effect of 0.06 M sodium succinate and 0.3 M ethyl carbamate on oxygen uptake of post-diapause intact embryos, their homogenates, supernatant (cytoplasm), and nuclei. First 60 minutes in Ringer. Arrow indicates time of emptying side arms into respiration flasks.

reagents. Inasmuch as no succinic dehydrogenase activity has been found for the nucleus of the grasshopper embryonic cells, it seems reasonable to assume that the action of urethane is not specific for that enzyme.

3. EFFECT OF SODIUM SUCCINATE AND 2,4-DINITROPHENOL

Previous reports have shown that 2,4-DNP exerts a marked stimulation of endogenous oxygen uptake of intact embryos and has little, if any, effect upon homogenates or intracellular constituents (Bodine, 1950). Succinate-treated intact embryos when subjected to 2,4-DNP show the characteristic stimulation of oxygen uptake due solely to the 2,4-DNP (Fig. 11). On the other hand, 2,4-DNP added to succinate-treated homogenates or intracellular constituents produces no changes in endogenous or succinate-stimulated oxygen uptake. Results for typical experiments are graphically shown in Figures 11 and 12.

4. EFFECT OF SODIUM SUCCINATE AND TRIPHENYLTETRAZOLIUM CHLORIDE

Triphenyltetrazolium chloride has been suggested as a more or less specific

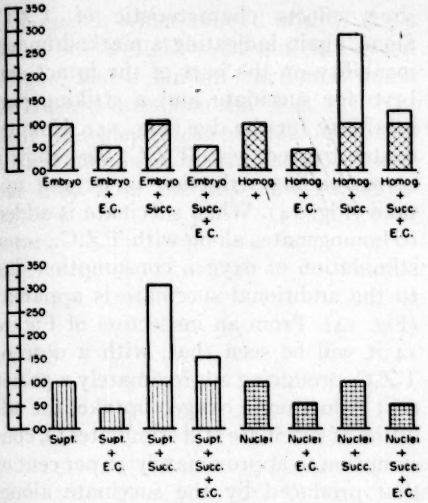

FIG. 10.—Same as Figure 9, except relative percentage effects of reagents shown.

vital stain for the detection of succinic dehydrogenase (Kun and Abood, 1949). When added to intact embryos and homogenates, it appears to stain them pink, indicating a reduction of the dye by some intracellular reducing system. That it may interfere with the normal workings of the inherent dehydrogenase systems seems reasonable if one assumes it to compete for the H^+ normally involved in dehydrogenase activity. When the dye is added to intact embryos or homogenates in increasing concentrations, a marked reduction in endogenous oxygen uptake occurs (Fig. 13). The reduction of the stain can be readily followed by noting the production of the pink reduced dye as well as by its quantitative extraction and estimation by spectrophoto-

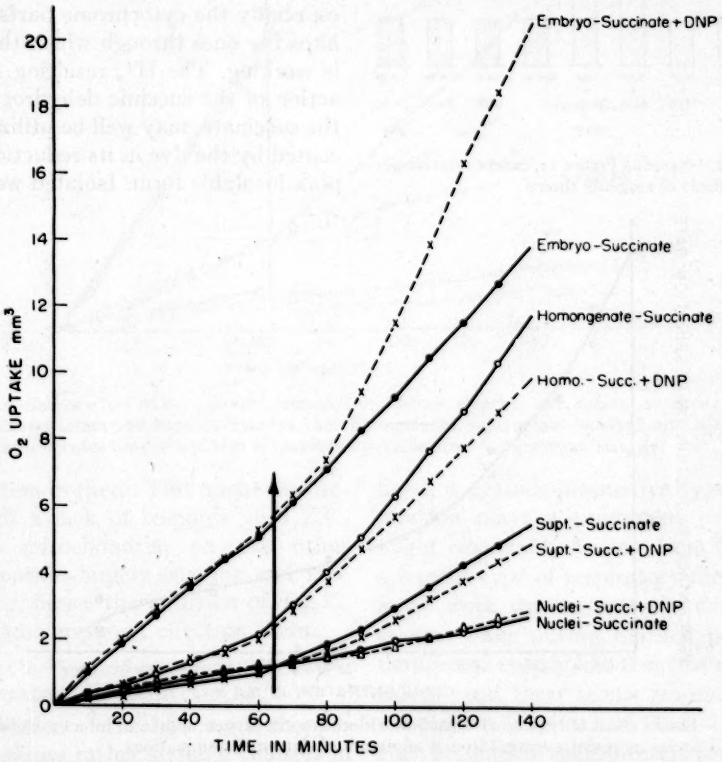

FIG. 11.—Shows effect of 0.06 M sodium succinate and 1×10^{-5} M, 2,4-dinitrophenol on oxygen uptake of diapause intact embryos, homogenates, supernatant (cytoplasm), and nuclei. Otherwise as in Figure 9.

metric methods. From an inspection of Figure 13 it can be seen that a concentration of 0.1 per cent produces a reduction in endogenous oxygen uptake of approximately 50 per cent. Intact embryos treated with both succinate and T.Z.C. show effects characteristic of T.Z.C. alone, again indicating a marked impermeability on the part of the intact embryo for succinate and a striking permeability for the dye (Fig. 14). Homogenates treated with T.Z.C. also show a corresponding reduction in oxygen uptake (Fig. 14). When succinate is added to homogenates along with T.Z.C., some stimulation of oxygen consumption due to the additional succinate is apparent (Fig. 14). From an inspection of Figure 14 it will be seen that, with a dose of T.Z.C. producing approximately a 50 per cent reduction in oxygen uptake, the addition of succinate still stimulates O_2 consumption to approximately 50 per cent of that produced by the succinate alone. From such a reaction one might well assume that the succinoxidase system and especially the cytochrome parts are perhaps the ones through which the T.Z.C. is working. The H^+, resulting from the action of the succinic dehydrogenase on the succinate, may well be utilized or accepted by the dye in its reduction to the pink insoluble form. Isolated washed in-

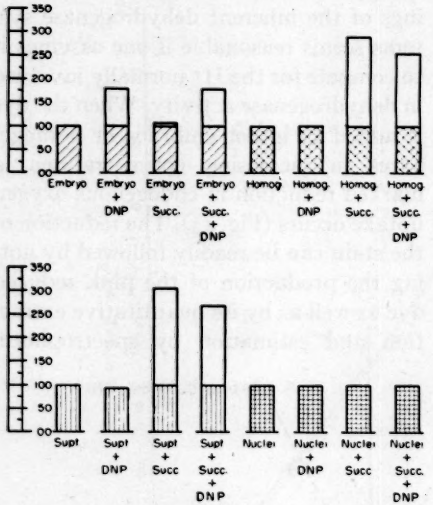

FIG. 12.—Same as Figure 11, except relative percentage effects of reagents shown.

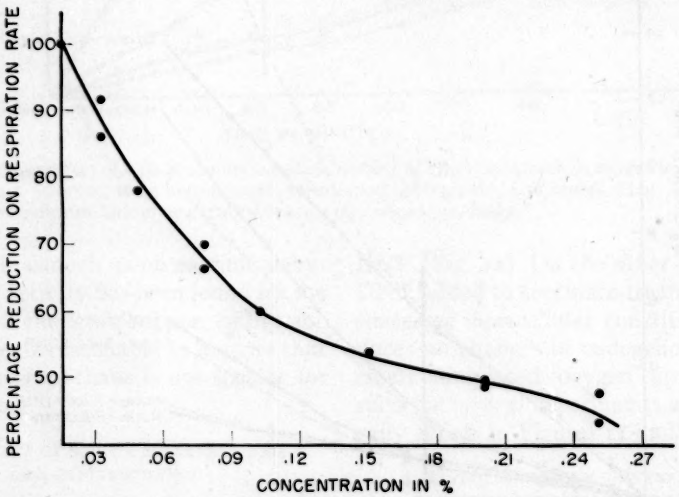

FIG. 13.—Shows effect of triphenyltetrazolium chloride upon oxygen uptake of intact postdiapause embryos. Solid circles represent averages from a minimum of sixteen determinations.

tact nuclei, when treated with T.Z.C., suffer but a slight reduction in oxygen uptake, and a similar reaction is noted for washed mitochondria (Fig. 14). Nuclear homogenates, as pointed out above, are not stimulated by succinate and hence one may infer the lack of a succinoxidase system in them. This might also account for a lack of response to T.Z.C. Washed mitochondria, on the other hand, contain largely enzyme and lack substrate; hence the addition of T.Z.C. produces no apparent effect on them.

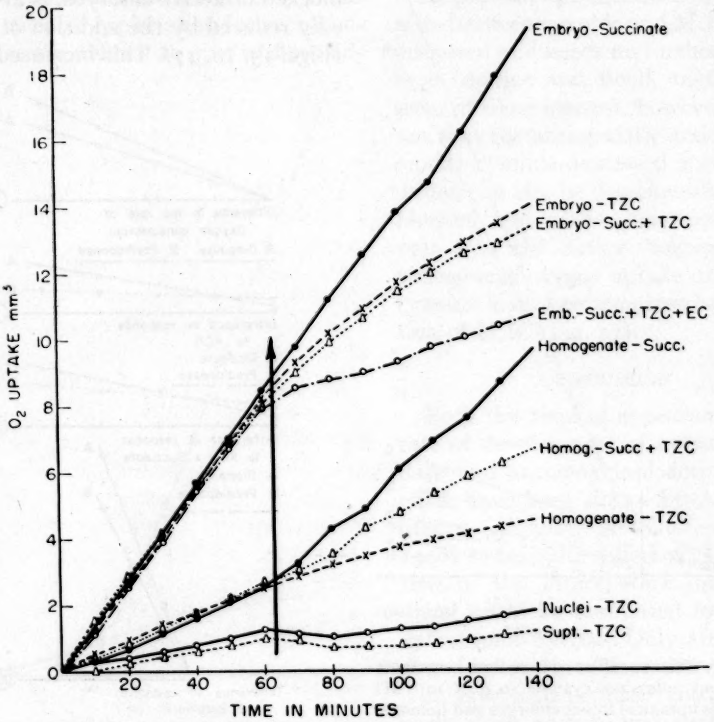

FIG. 14.—Shows effect of 0.1 per cent triphenyltetrazolium chloride and sodium succinate upon the oxygen uptake of intact postdiapause embryos, their homogenates, supernatant (washed mitochondria), and nuclei. Arrow indicates time of addition of reagents from side arm to respiration flask.

5. EFFECTS OF SODIUM SUCCINATE, POTASSIUM CYANIDE, AND METHYLENE BLUE

Respiration of the intact grasshopper embryo shows rather striking changes in its sensitivity to KCN during the course of development at 25° C. (Bodine and Boell, 1934). A cyanide-sensitive fraction seems to play a dominant role during stages of active growth and cell division, namely, pre- and postdiapause. During mitotic block or diapause, on the other hand, a cyanide-insensitive type of respiration plays a prominent part. One might reasonably assume from this that a ferrous type of respiratory mechanism is at work during actively developing stages, while during blocked periods a nonferrous type holds. If such a situation exists—and there seems reasonable evidence for it (Bodine and Boell, 1934)—it then becomes of some interest to compare

the action of such reagents as cyanide and methylene blue (M.B.) upon the succinoxidase system during these periods. Since the intact embryo is impermeable to succinate, it obviously is impossible to test the effects of these reagents except upon the homogenates derived from the embryos.

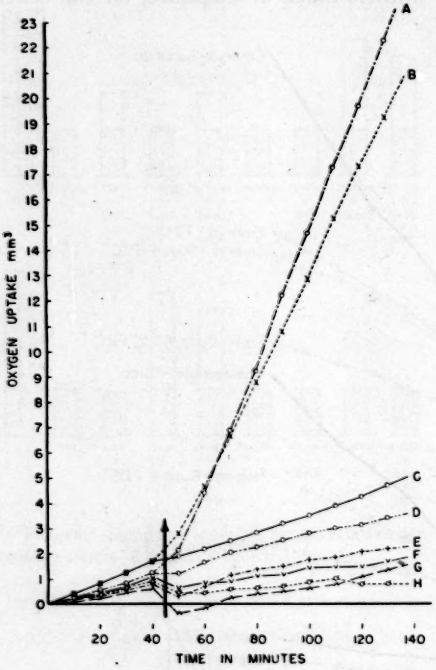

FIG. 15.—Shows effect of sodium succinate (0.06 M) and potassium cyanide (0.46×10^{-3} M) upon oxygen uptake of intact embryos and homogenates suspended in 0.25 M sucrose. Arrow indicates time of addition of reagents from side arm to respiration flask. *A* = diapause supt. in sucrose, succinate added; *B* = prediapause supt. in sucrose, succinate added; *C* = prediapause control, succinate added; *D* = diapause control; *E* = diapause, KCN + succinate; *F* = prediapause, KCN + succinate; *G* = diapause, KCN; *H* = prediapause, KCN.

Potassium cyanide added to homogenates of pre- or postdiapause embryos produces marked lowering effects upon the endogenous oxygen uptake. Similar concentrations added to diapause homogenates, on the other hand, produce little, if any, effect. Typical results of such experiments are graphically shown in Figures 15, 16, and 17. Stimulation of oxygen uptake, due to the addition of succinate to homogenates from either blocked or active embryos, is also markedly reduced by the addition of cyanide (Figs. 15, 16, 17). This increased O_2, due to succinate, is apparently quite cyanide-sensitive, irrespective of the physiological states of the embryos from which the homogenates are derived. In view of such reactions it seems reasonable to assume that the workings of the succinoxidase

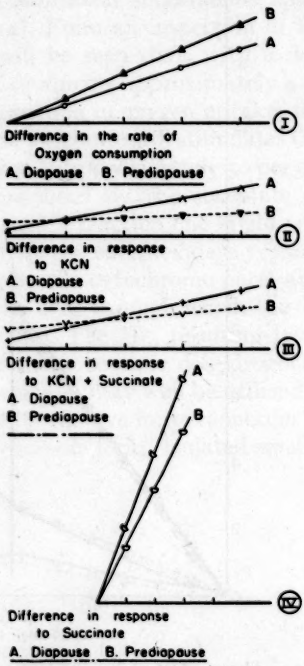
Difference in the rate of Oxygen consumption
A. Diapause B. Prediapause
Difference in response to KCN
A. Diapause
B. Prediapause
Difference in response to KCN + Succinate
A. Diapause
B. Prediapause
Difference in response to Succinate
A. Diapause B. Prediapause

FIG. 16.—Same as Figure 15 but shows diagrammatically the relative effects of cyanide and succinate upon diapause and prediapause homogenates. Abscissa time in 20-minute periods: ordinates relative O_2 uptake. Slopes of lines indicate differences in rates of O_2 uptake.

system of the different homogenates are similar and that the cyanide inhibition must be largely through its effect upon the cytochrome system. For the diapause homogenate the succinate-stimulated oxygen uptake is quite cyanide-sensitive, while the endogenous oxygen uptake seems cyanide-insensitive (Figs. 15 and 16). A suitable explanation for such a reaction seems difficult to give without additions or modifications of orthodox concepts of the *modus operandi* of succinoxidase. Since cytochrome C and cytochrome oxidase appear to be present in both types of homogenates, one might reasonably assume some different enzyme systems to explain the reactions of the blocked homogenates. Further work on these points is in progress and will be reported at a later date.

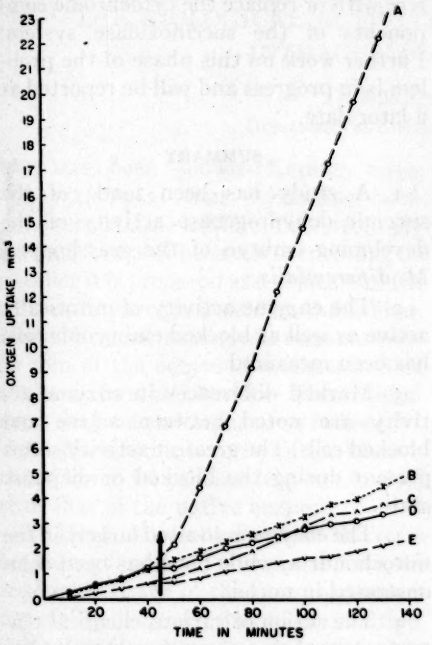

FIG. 17.—Shows effect of succinate, cyanide, and methylene blue upon oxygen uptake of postdiapause supernatant (cytoplasm without nuclei) suspended in 0.25 M sucrose. Note absence of effect of M.B. upon supernatant, while a striking antagonism is shown between KCN and M.B. Arrow indicates time of addition of reagents from side arm to respiration flask. *A* = supt. in sucrose, succinate added; *B* = supt. in sucrose, M.B. added; *C* = supt. in KCN + sucrose, M.B. added; *D* = supt. in sucrose, control; *E* = supt. in KCN + sucrose, control.

In previous publications the antagonism between cyanide and M.B. has been discussed and needs no further comment here (Bodine and Boell, 1936). It does seem of some interest, however, to point out that the antagonistic action of M.B. on the cyanide-depressed succinate oxidation can also be demonstrated for both blocked and active homogenates (Fig. 17). For the active homogenate the endogenous oxygen uptake depressed by cyanide is also antagonized by the addition of M.B. (Fig. 17).

DISCUSSION

Both the time of appearance and the rate of development of cytochrome oxidase and succinoxidase activity in the chick have been shown by Albaum and others (Albaum, Novikoff, and Ogur, 1946) to be quite different. The respiration of the intact chick embryo (1–3 days) could be accounted for solely by cytochrome oxidase. Only after the fifth day does succinoxidase activity appear. The low succinoxidase activity in the early chick embryo was explained by a low limiting concentration either of succinic dehydrogenase or of another system which links the succinic dehydrogenase to the cytochrome oxidase system. In the grasshopper embryo the development of the cytochrome oxidase system closely parallels the respiration curve throughout (Allen, 1940). The succinoxidase activity, on the other hand, is low in early prediapause and slowly increases up to the time of diapause. During the marked fall in respiration at the onset of dia-

pause, succinoxidase activity increases at a rapid rate and reaches a maximum, which is maintained throughout the entire diapause or blocked period. When diapause is broken, a corresponding drop in succinoxidase activity takes place, and this is followed in postdiapause by a very gradual increase up to the time of hatching. This increase in succinoxidase activity is rather difficult to explain, since, during diapause, respiration seems to be largely of a cyanide-insensitive type, suggesting other than the usual cytochrome system as being active. In a subsequent communication additional data on the cytochrome systems of the embryonic cell of the grasshopper are to be presented.

Studies on the localization of the succinic dehydrogenase in the embryonic cell of the grasshopper seem to place it in the mitochondria, and in this situation it resembles that found for other biological forms (Lardy, 1949). Inhibition of succinoxidase activity in the embryo of the grasshopper is also produced by narcotics like ethyl carbamate. Reagents like 2,4-DNP which stimulate the oxygen uptake of intact embryos do not seem to act upon the succinoxidase system of the homogenates formed from embryos. The dye triphenyltetrazolium chloride, as indicated above, apparently exerts an inhibitory action upon the oxygen uptake of both intact and homogenized embryos. This inhibition for the homogenate seems to be associated with the succinoxidase system, since, when both dye and succinate are added to homogenates, marked reduction in the succinate-stimulated oxygen uptake is noted. Since the dye is rapidly and irreversibly reduced by both homogenate and intact embryo, it seems reasonable to assume that it may act as a hydrogen acceptor and in this way interfere with or replace the cytochrome components of the succinoxidase system. Further work on this phase of the problem is in progress and will be reported at a later date.

SUMMARY

1. A study has been made of the succinic dehydrogenase activity of the developing embryo of the grasshopper, *M. differentialis*.

2. The enzyme activity of mitotically active as well as blocked embryonic cells has been measured.

3. Marked differences in enzyme activity are noted between active and blocked cells. The greatest activity seems present during the blocked or diapause state.

4. The enzyme is located largely in the mitochondria, while none has been demonstrated in nuclei.

5. The action of various chemical reagents upon the succinoxidase activity has been studied.

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THE BIOLOGICAL ACTIVITY AND PHYSICAL STATE OF INTRACELLULAR CATALASE[1]

J. GORDIN KAPLAN[2]
Department of Zoölogy, Columbia University

IT HAS been shown (Kaplan, 1950, 1951, 1952) that crystalline beef-liver catalase exhibits two strikingly different kinds of behavior, depending on whether it is prepared as a solution of the native protein or as a solid insoluble fiber, formed by compression of a monomolecular film of the enzyme protein adsorbed at the air/water interface. These differences may be summarized as follows:

A. The specific activity of the fibers is much lower (approximately one-fourth) than that of the native enzyme.

B. Heat, of relatively short duration between 50° and 56° C., can increase the residual activity of the fiber, whereas it invariably decreases that of the solution.

C. The fiber is much more resistant to inactivation by heat of longer duration or at higher temperature than is the native enzyme. Figure 1 illustrates these differences with respect to heat and is included to permit comparison with the data presented below.

D. The activity of the fiber is much less sensitive to ultraviolet irradiation at 254 mμ than is that of the solution of the native protein from which it was prepared.

The experiments presented in this paper were designed to permit comparison of the behavior of the intracellular catalase of beef erythrocytes to that of the same enzyme extracted from the cell into solution. The intracellular and extracted enzymes were confronted with the same experimental conditions which had revealed the differences in biological activity of the surface-denatured and native catalase summarized above. Close agreement in activity of the fibrous and intracellular catalases would suggest that they share some common property of configuration or physical state.

MATERIAL AND METHODS

Fresh defibrinated beef blood was used within 3 days after its removal from the animal. Pyrex-redistilled water and McIlvaine's phosphate-citrate buffer at pH 6.9 were employed throughout. Activity measurements were made manometrically with the circular Warburg-Barcroft apparatus, which permits very rapid readings. A 0.06 per cent solution of H_2O_2

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[2] Present address: Department of Physiology, Dalhousie University, Halifax, N.S.

made up in isotonic (0.15 M) NaCl was the substrate, 1 ml. being added to the side arms of the Warburg vessels and tipped in after 10 minutes' equilibration at 30° C. All preparative procedures were carried out in a 5° C. cold room.

1. INTRACELLULAR ENZYME

For the preparation of the intracellular enzyme, a 1-ml. sample of the blood was diluted with isotonic saline; the cells were then separated from the plasma by

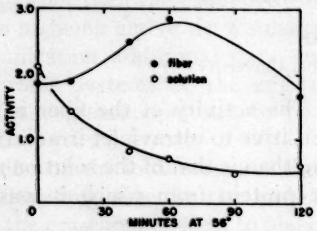

FIG. 1.—Activities of fibers and solutions after varying durations at 56° C. Each point represents an average of four separate determinations.

centrifugation and washed four times. Finally, the erythrocytes were covered with 50 ml. of saline and 15 ml. of buffer, the mixture constituting the *whole-cell suspension*. One-ml. samples were then pipetted into Warburg flasks for activity measurements.

2. EXTRACTED ENZYME IN SOLUTION

Another 1-ml. sample of blood was simultaneously prepared exactly as above, except that, following the fourth washing, 25 ml. of water, 25 ml. of twice isotonic (0.30 M) saline, and 15 ml. of buffer were added in that order; before the addition of the last two components, the cells were stirred until hemolysis was complete. The supernatant after a final centrifugation constitutes the *hemolysate*, which is a clear solution of hemoglobin and other intracellular substances, including catalase. The stroma were found to be neutral as far as catalase activity was concerned.

3. SPECIFIC ACTIVITIES AND THE EFFECT OF PHYSICAL AGENTS

The activities of the whole-cell suspension and hemolysate can now be directly compared, as a given volume of each should contain the same absolute amount of enzyme in medium of equal salinity and pH. The whole-cell suspension under these conditions decomposes its substrate at a constant rate, following a brief lag period, which is doubtless due to the necessity of establishing a steady state. The linearity of the curves (see Fig. 2, *b* and *b'*), indicating independence of substrate concentration, is presumably due to saturation of the intracellular enzyme; the question of the permeability of the red-cell membrane is thus minimized.

To study the effect of heat, both mixtures were incubated in a constant-temperature bath of high precision (±0.01° C.), and aliquots were withdrawn as desired. To subject samples to ultraviolet irradiation, 10-ml. samples of each were pipetted into Syracuse dishes placed 25 cm. below a mercury-vapor tube delivering 85 per cent of its energy at the 254 mμ line. The cells were stirred at frequent intervals to prevent them from settling and adhering to the glass.

4. THE CONTROL OF HEMOLYSIS

Both heat and ultraviolet light cause hemolysis of intact cells. Consequently, these agents, when acting on the whole-cell suspension, cause the extraction of the catalase from the cell, thus making unclear the distinction between the cell suspension and the hemolysate. Therefore, whenever aliquots of the whole-cell suspension were withdrawn for activity assay, an additional 5-ml. sample was pipetted into a centrifuge tube and spun at 3,000 r.p.m. for 10 minutes, causing all

the intact cells to come down. The *supernatant*, containing whatever hemoglobin and enzyme had been extruded, was collected; the *centrifugate* was washed with isotonic saline and covered with 5 ml. of this fluid, thus bringing back the cells to what was their approximate concentration before centrifugation. One-ml. samples of both supernatant and diluted centrifugate were then tested for activity and compared to that of the uncentrifuged whole-cell suspension. Essentially,

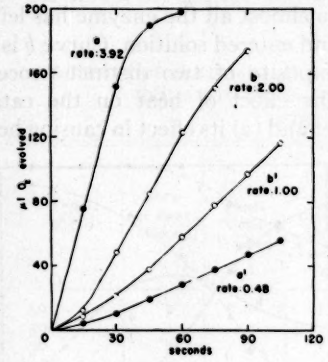

FIG. 2.—Activities of: *a*, hemolysate at 0 hours, *a'*, after 4 hours at 56°; *b*, whole-cell suspension at 0 hours, *b'*, after 4 hours at 56° C.

then, for each measurement of the activity of the whole-cell suspension, comparable activity measurements of the cells alone and the supernatant alone were also made, to determine the degree of hemolysis caused by the heat treatment and the activity attributable to the nonhemolyzed cells alone.

The supernatant was also tested in the Klett photoelectric colorimeter with a green filter: comparison of the optical density of this fluid with that of the original hemolysate indicates the degree of hemolysis of whole cells at any point in the experiment. Thus the effect of physical agents in causing extraction of the intracellular enzyme can be clearly distinguished from their effects directly on the catalase within the cell; the activity of the centrifugate can be corrected to account for those cells which have hemolyzed and whose activity has therefore passed to the supernatant. This correction is necessary for demonstration of a possible heat activation of the intracellular enzyme, and it is justified, since the expected linear relationship between cell number and catalase activity has been experimentally confirmed.

RESULTS

1. SPECIFIC ACTIVITY

Figure 2, curves *a* and *b*, shows the activity of a whole-cell suspension compared to that of a hemolysate, originally consisting of the same number of cells;

TABLE 1

ACTIVITY OF WHOLE-CELL SUSPENSION AND HEMOLYSATE
(Standard Deviations Shown)

Material	No. of Samples	Average Activity
Hemolysate (*a*).....	26	4.33±0.60
Whole-cell suspension (*b*)	28	2.10±0.29

Using matched pairs (i.e., prepared from same blood sample): Ratio of activities (a/b) = 2.10 ± 0.33.

the hemolysate possesses twice the activity of the intact cells. Table 1 summarizes the data for specific activities of the enzyme inside and outside the cell; the hemolysates are twice as active. The intracellular catalase seems, therefore, to be in a less active condition than when it is brought out of the cell into solution.

2. EFFECT OF HEAT

a) *Resistance to heat inactivation.*—Figure 2, curves *a'* and *b'*, shows the decrease in activity of both preparations following heating for 4 hours at 56°. The extracted enzyme is clearly much more easily inactivated.

The time course of changes of the various preparations where the temperature was maintained constant at 56° is shown in Figure 3. Curve *b*, the whole-cell suspension, at 0 hours has but half the activity of the hemolysate, but, upon being heated, it rapidly increases in activity and soon surpasses the hemolysate; upon reaching a maximum, it falls off, in this experiment at a rate somewhat slower than curve *a*, but in other experiments at exactly the same rate. Following the intersection of curves *a* and *b* at about the 1-hour point, curve *b* demonstrates that the intracellular enzyme is well protected from heat inactivation, since its activity continues to rise. Curve *c* represents the washed centrifugate, i.e., whole cells only. At 0 hours, 100 per cent of the activity of the whole-cell suspension is in the cells, but, with heating, less and less activity is recovered in the cell, until at 3 hours all has virtually disappeared, since almost all the cells have been hemolyzed by this time. Curve *d* shows the activity of the supernatant, which is 0 at 0 hours, increases to a maximum at 2 hours, and thereafter falls off along curve *b*, since almost all the activity of the whole-cell suspension is then in the supernatant.

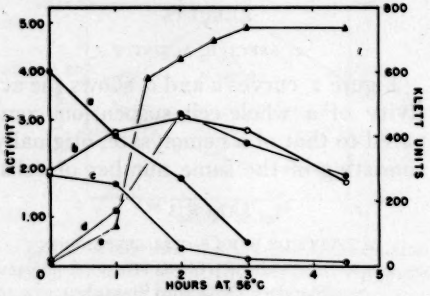

FIG. 3.—Curves *a*, *b*, *c*, and *d* represent activities as follows: *a*, of hemolysate; *b*, of whole-cell suspension; *c*, of centrifugate (cells alone); *d*, of supernatant. Curve *e* = optical density of supernatant.

Curve *e* represents the optical-density measurements of the supernatant; at 0 hours there is no absorption by the supernatant, indicating absence of hemolysis; but at 3 hours 100 per cent hemolysis has been reached. Part of the increase in activity of the whole-cell suspension (curve *b*) is therefore due to the progressive heat hemolysis of the whole cells, which liberates the enzyme in a more active condition; the activity of the whole-cell suspension decreases only at the point where hemolysis is practically complete and where almost all the enzyme has left the cell and entered solution. Curve *b* is thus a composite of two distinct processes: (1) the effect of heat on the catalase proper and (2) its effect in causing hemolysis of the cells, thus temporarily increasing the activity of the whole mixture but rendering it labile to heat inactivation.

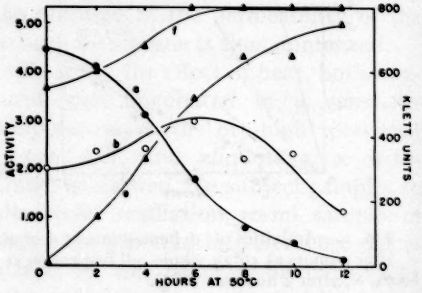

FIG. 4.—Curves *a* and *b* represent activities as follows: *a*, of hemolysate; *b*, of whole-cell suspension. Curves *e* and *f* represent optical densities as follows: *e*, of supernatant of whole-cell suspension; *f*, of centrifuged hemolysate.

Figure 4 summarizes the results for a similar group of data, but with the temperature maintained at 50°; these are qualitatively quite similar to those at 56°. Curve *a* shows the decrease in activity of the hemolysate, curve *b* the initial increase in activity of the whole-cell suspension; the displacement of curve *b* to the right following intersection with

curve *a* again demonstrates unequivocally the resistance to heat inactivation of the intracellular enzyme. Curve *e* represents the optical density of the supernatant, showing almost complete hemolysis at 8 hours. Curve *f* shows the optical density of the hemolysate; the increase in absorption, correlated with its change in color from a clear red to a dark, brownish shade, is a consequence of the oxidation of hemoglobin to methemoglobin.

b) *Heat activation.*—Using the method already described, the optical densities of the supernatants of whole-cell suspension and hemolysate were compared for varying duration of heating at both 50° and 56°; from the per cent hemolysis thus obtained, a factor (Table 2, col. *d*) is derived which, when applied to the activity of the centrifugate (intact cells), corrects for the number of cells lost by heat hemolysis. This corrected activity can then be compared to that of the unheated controls (i.e., the centrifugate at 0 hours), and any difference between these activities is a measure of the effect of heat on the enzyme still remaining within the cells.

The data are summarized in Table 2.[3]

[3] Figures 3 and 4 are not intended to bear on the question of heat activation.

Comparison of columns *e* and *f* shows that the corrected activities of the cells heated for relatively short durations at 50° and 56° are in all cases higher than those of the unheated controls. Therefore, heat activation of the intracellular enzyme is also partly responsible for the increase in activity of the heated whole-cell suspensions.

3. EFFECT OF ULTRAVIOLET IRRADIATION

Figure 5 illustrates the effect of UV on the hemolysate and on whole cells; it is strikingly similar to that of heat, although no detectable increase in temperature occurs beneath the UV source used. Small local rises in temperature must be ruled out as an explanation for the effect of UV, since cells may be maintained for 4 hours at a temperature as high as 45° without causing any hemolysis. Curve *a*, the hemolysate, shows a rapid decrease in activity to about 50 per cent of initial activity after 9 hours of irradiation. At this point, the whole-cell suspension still possesses more activity than it had at 0 hours. The intracellular catalase is therefore quite insensitive to UV irradiation, but becomes labile upon extraction from the cell. Curve *e*, colorimeter readings on supernatants, shows that UV causes

TABLE 2

ACTIVITY OF CENTRIFUGATE OF WHOLE-CELL SUSPENSION, CORRECTING FOR HEMOLYSIS: DEMONSTRATION OF HEAT ACTIVATION OF INTRACELLULAR CATALASE

Temp. (° C.)	Dura- of Heat (Hours)	No. Times Cells Washed (*a*)	Activity of Centrifugate Uncorrected (*b*)	Per Cent Hemolysis (*c*)	Correction Factor (*d*)	Activity Corrected (*e*)	Activity of Unheated Centrifugate (*f*)
50.....	2	1	1.56	25	100/75	2.08	1.56
50.....	4	1	1.44	49	100/51	2.82	1.56
50.....	2	4	1.30	39	100/61	2.13	1.87
56.....	1	1	1.72	19	100/81	2.12	1.84

hemolysis, while curve *f* shows that UV can cause the oxidation of hemoglobin.

DISCUSSION

1. COMPARISON OF SURFACE-DENATURED AND INTRACELLULAR CATALASE

a) *Specific activity.*—The specific activity of the intracellular enzyme has been shown to be half that of the same enzyme extracted from the lysed cell. Similarly, it has been demonstrated that the activity of a surface-denatured preparation decreases to about one-fourth that of the original solution of native enzyme.[4]

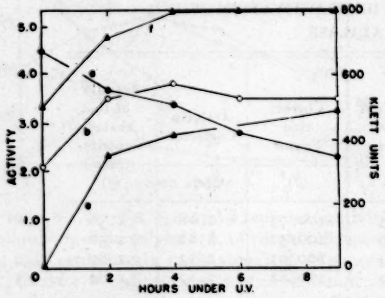

FIG. 5.—Curves *a* and *b* represent activities as follows: *a*, of hemolysate; *b*, of whole-cell suspension. Curves *e* and *f* give optical densities as follows: *e*, of supernatant of whole-cell suspension; *f*, of centrifuged hemolysate.

Unfortunately, the possibility that the lower activity of the intracellular enzyme is a permeability effect cannot be eliminated on the basis of the present data. The apparent zero-order kinetics exhibited by the intracellular enzyme-substrate system have been interpreted as demonstrating the saturation of the enzyme. An alternate, if less likely, explanation is that the cell membrane itself becomes "saturated" with the substrate and allows the penetration of the hydrogen peroxide molecules at a slow but constant rate, thus preventing the saturation of the enzyme. Experiments designed to test this alternate explanation are in progress.

The increase in activity of various enzymes and enzyme systems following cytolysis is widely known and has been found in the case of the catalase of sea-urchin eggs by Doyle (1938). The usual interpretation of this phenomenon is that the intracellular enzyme is in a "blocked" or partially "masked" condition. Another explanation will be advanced below.

b) *Heat activation.*—It has been demonstrated that heat at 50°–56° can increase the residual activity of the surface-denatured catalase, but not that of the original solution of the native enzyme. That application of a prior heat treatment will increase the catalase activity of a variety of cells and crude extracts has been widely reported. Destruction of a heat-labile "inhibitor" (the anti-catalase of Stern, 1927) was postulated to explain this anomalous result in the case of erythrocytes. The existence of such an "inhibitor" is contradicted by the present data, since heat uniformly decreases the activity of the enzyme in solution (hemolysate) and can increase *only* the activity of the whole-cell suspension. In any case, the effects of heat which the "inhibitor" had been invoked to explain have been shown to be due both to hemolysis of the cells and to activation of the enzyme still remaining within the cells.

Bodnár and Bártfai (1936) report that if bacon fat is heated for 2 hours at from 31° to 45° (depending on whether it is summer or winter fat), its catalase activity may be increased up to 112 per cent. Stern (1927) found that catalase activity of erythrocytes is greatly increased by heat at 60°. Euler and Blix (1919), working with yeast cells, showed that catalase

[4] All references to the enzyme *in vitro* are to the publications previously cited (Kaplan, 1950, 1951, 1952).

activity could be greatly increased by preheating the cells for ½–2 hours at temperatures ranging from 55° to 63°, with an optimum at 58°. They claim that they obtained the same results with a variety of other microörganisms, but not with one variety of yeast (Euler and Laurin, 1919). Euler and Borgenstam (1920) repeated this work with horse erythrocytes and found increases in catalase activity of up to 170 per cent after heating at 57°; they call attention to the close agreement between the maxima for heat activation of yeast and blood cells. They conclude that the cause of this phenomenon must be sought in the physical condition of dispersion (*Dispersitätsgrade*) of the enzyme within the cell. Nosaka (1928) likewise observed an increase in activity of erythrocyte catalase upon heating; he attributed his results, as well as those of Euler and Borgenstam, to hemolysis caused by heat—a factor neglected by those authors. The present data show that most of the increase in activity of a heated suspension of blood cells is indeed due to hemolysis, as stated by Nosaka; yet the activity of heated, nonhemolyzed whole cells has also increased, showing that not all the increase can be ascribed to hemolysis but that a part must be due to an actual heat activation of the intracellular enzyme.

c) *Resistance to heat inactivation.*—Surface-denatured catalase has been shown to be quite resistant to heat inactivation as compared to the solutions from which it was prepared (Fig. 1). As long as the enzyme remains within the cell, it, too, has been proved to be highly resistant to heat inactivation; but after hemolysis and upon entering solution, it becomes highly labile to destruction by this agent.

d) *Ultraviolet irradiation.*—It has been shown previously that a 4-hour UV irradiation of a solution of crystalline catalase abolished virtually all activity, in contrast to which the surface-denatured enzyme retains approximately 60 per cent of its activity under the same conditions. The present data, as seen in Figure 5, show that the intracellular enzyme is likewise stubbornly resistant to inactivation by UV, as opposed to the same enzyme removed from the cell.

The stability of intracellular catalase to UV reported here receives strong corroborative support from the literature. Euler (1933) has reported that UV-treated yeast cells possessed more catalase activity than did his unirradiated controls. Frenkel (1949), working with *Chlorella*, found that an exposure to UV which would completely stop all photosynthesis would result in a several fold increase in catalase activity.

Stewart and Aldous (1951) have irradiated yeast cells with intensities of UV high enough to permit but 5 per cent survival. They then dried the cells and prepared enzyme extracts from the powder. They found carboxylase and hexokinase to be UV-sensitive, the former retaining roughly 35 per cent of its activity as compared to the unirradiated controls. On the other hand, catalase (as well as lactic and alcohol dehydrogenases) was completely insensitive to UV.

2. PHYSICAL STATE OF THE INTRACELLULAR ENZYME

To account for these data, it has been assumed that the surface-denatured and the intracellular catalases both differ in the same direction from a solution of this enzyme, in that they possess in common some aspect of physical structure or configuration not shared by the native molecule. Our working hypothesis states that catalase within the cell exists partially, but reversibly, unfolded at an intracellular interface (see par. *e*, below); upon cytolysis, the enzyme is desorbed from

the interface and changed to the rolled-up (native), soluble condition, with consequent increase in activity and greater sensitivity to heat and UV. The evidence in favor of this hypothesis is presented below.

A. The experiments showing a point-for-point similarity in biological activity of surface-denatured and intracellular enzymes as opposed to solutions of the native catalase, as well as what is known about the localization of catalase within the mammalian liver cell (Chantrenne, 1944), seem completely to preclude the possibility that the intracellular enzyme exists in the "native," soluble state.

B. Examination of the literature has shown with a variety of cell types that the intracellular enzyme shares the properties of the surface-unfolded catalase, in so far as these points of difference from the native enzyme are concerned.

C. Working with intact cells, Kopac (1950) reports that "cytoplasmic proteins undergo little, if any, surface denaturation at experimentally introduced oil-water interfaces provided the cytoplasm remains intact. If the cell is cytolyzed within 30 to 60 seconds after the injection of an oil drop into intact cytoplasm, the surface-denaturation of the released proteins is so extensive that the entire interfacial area becomes occupied by protein molecules in various stages of unfolding. The rapid adsorption of proteins and subsequent unfolding causes the interfacial area to expand, thereby producing a crinkled interface—the Devaux effect." Cytolysis is thus accompanied by changes in the state of cellular proteins, permitting them to spread at the interface presented by the injected oil drop.

D. An alteration in the physicochemical state of proteins following cytolysis has been hypothesized by Vlès and Gex (1928). These authors, using a quartz microscope in the ultraviolet and paying particular attention to the protein band at 275 mμ, found that the absorption curve of the intact, living sea-urchin egg was quite different from that of a cell homogenate in aqueous medium but that, following cytolysis, the same cell showed an absorption picture similar to that of the aqueous suspension.

E. The type of interface required by the present hypothesis is such as might exist between oil and water with surface-active molecules or groups present in either or both phases. Proteins will not become irreversibly denatured at such an interface, provided that the tension between the two phases be sufficiently low (Danielli and Harvey, 1934); the observed oil/water interfacial tension within the cell is strikingly low—of the order of 1 dyne/cm (Harvey and Shapiro, 1934). Jacques Loeb (1913) and more recent authors (Monné, 1948) have emphasized the central role of the separation of fats from proteins in the cytolytic process.

Experiments to test this working hypothesis, with reference to the behavior of proteins at the oil/water interface and to the biological activity of intracellular enzymes, are in progress.

SUMMARY

1. The enzyme catalase within the cell differs from a solution of the enzyme extracted after lysis of the cell in the following ways: (*a*) Its specific activity is considerably lower; (*b*) heat at 50° or 56° C. will initially increase its residual activity, whereas it decreases uniformly the activity of the extracted enzyme; (*c*) it is far more resistant to inactivation by heat at higher temperatures or longer durations; and (*d*) it is quite resistant to intensities of ultraviolet irradiation to which the extracted solution is sensitive.

2. The foregoing four criteria have already been used to differentiate the bio-

logical activity of surface-unfolded catalase from that of the solution of protein from which it was prepared. The surface-unfolded and intracellular catalases behave similarly as far as these four points are concerned.

3. The working hypothesis advanced to account for these facts is that the enzyme within the cell is in a reversibly unfolded condition. Upon cytolysis or extraction from the cell, it becomes desorbed from the intracellular interface and enters solution, assuming the rolled-up, globular configuration, with consequent increase in activity and instability to heat and ultraviolet. The evidence on which this hypothesis is based is discussed.

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THE OCCURRENCE OF THIAMINASE IN CERTAIN FRESH-WATER FISHES

ALFRED B. CHAET[1] AND DAVID W. BISHOP

Department of Zoölogy, University of Massachusetts, Amherst

THE enzyme thiaminase functions in the hydrolytic cleavage of the thiamine (vitamin B_1) molecule, splitting it into its two major components, thiazole and pyrimidine. Certain thiaminase-containing fishes present in the food given to foxes has been the cause of vitamin B_1 deficiency (Chastek paraly-

[1] Present address: Department of Zoölogy, University of Pennsylvania, Philadelphia.

sis) in the animals (Green, 1937). Interest in thiaminase has recently increased for two reasons. In the first place, the enzyme appears to be important from a nutritional and enzymological standpoint; and, second, it can perhaps be used in determining phylogenetic relationships. Of the 138 forms assayed for the presence of thiaminase, 66 contain the enzyme (Chaet, 1950; Harris, 1951). (Various species of fishes, particularly, have been investigated for the presence of thiaminase.) The distribution of the enzyme is not random, and a plausible explanation, though only a partial one, is the correlation with the animal's position in the animal kingdom. This paper adds further data on thiaminase distribution in 9 fresh-water species of fish and is reported for its bearing on the hypothesis that the distribution of the enzyme may be of significance as a phylogenetic characteristic.

PROCEDURE

The qualitative method used here for thiaminase determination is essentially a controlled incubation of the enzyme with a known amount of thiamine. The animal to be assayed was starved for 3 or more days to evacuate the intestinal tract. The whole fish was analyzed following maceration and extraction of the enzyme in 10 per cent NaCl (2 cc NaCl/gm wet weight of tissue). Ten cc. of this extract were added to 25 cc. (12.5 μgm.) of thiamine buffered at pH 4.5. The mixture was incubated at 37° C. for 24 hours, after which time the enzymatic reaction was halted by boiling. The remaining thiamine was separated from the mixture by adsorption on a synthetic zeolite ("Decalso"), eluted, and the concentration of thiamine determined by the thiochrome method (Association of Vitamin Chemists, 1947). Controls, containing 10 cc. of previously boiled extract, as well as standards of pure thiamine, were subjected to the same treatment.

Seven members of the family Cyprinidae and 2 of the Centrarchidae were investigated by this procedure.

RESULTS

The results show that thiaminase is present in all 7 cyprinids and absent in the 2 centrarchids assayed (Table 1). The results are significant in the light of thiaminase determinations reported for other species from these two families of fishes. Of the 23 cyprinid species which have been analyzed, 22 contain the enzyme. The one negative result, on the asp, is subject to question, as the muscle was excluded in the analysis, Sealock *et al.* (1943) having previously indicated in their work on carp that muscle tissue contains very little, if any, thiaminase. However, Gabrielsen (1950) has since shown that the muscle of herring, *Clupea harengus*, contains considerable quantities of thiaminase. All 8 members of the Centrarchidae so far assayed lack the enzyme. The presence or absence of the enzyme within these two families is thus very consistent.

DISCUSSION

The uniformity reported here for two families is substantiated by work on other groups of fishes. For example, the 6 members of the family Salmonidae analyzed lack thiaminase; the 3 representatives of the family Clupeidae all have it. On the other hand, there are a few families (e.g., Gadidae and Percidae) in which certain members have and others do not have the enzyme. The chance that this distribution is a random one is indeed slim. However, the meaning of this distribution, as Harris (1951) has pointed out, is not clear. There appears to be no single environmental factor or group of factors which determines the oc-

currence of thiaminase in an animal. It is hoped that an extension of such analyses to other species may further elucidate the biological significance of thiaminase distribution.

Certain general trends in the occurrence of thiaminase are of interest in that, while present in many fresh-water fishes, thiaminase is rare in most salt-water forms. Furthermore, of the 44 most advanced (phylogenetically) bony fishes analyzed—those stemming from the berycoid fishes—39 lack thiaminase. On the other hand, of the 66 forms reported to contain thiaminase, 61 are derived from ancestors more primitive than the berycoids. While thiaminase has been reported in a variety of invertebrates, it is absent in the few vertebrates, more advanced than the fishes, which have been investigated.

SUMMARY

1. Nine additional species of fish were assayed for the presence or absence of thiaminase. Seven species of the family Cyprinidae contain the enzyme, whereas

TABLE 1

OCCURRENCE OF THIAMINASE IN TWO FAMILIES OF FRESH-WATER FISHES

Species	Thiaminase	Reference
Family Cyprinidae:		
Abramis blicca	+	Lieck and Ågren, 1944
A. brama	+	Bhagvat and Devi, 1944; Ender and Helgebostad, 1945; Lieck and Ågren, 1944; Wiken and Ågren, 1947
A. vimba	+	Lieck and Ågren, 1944
Aspius aspius	+	Wiken and Ågren, 1945
A. rapax	−	Lieck and Ågren, 1944
Carassius auratus	+	Chaet, 1950; Deutsch and Hasler, 1943
C. carassius	+	Jacobsohn and Azevedo, 1947
Cyprinus carassius	+	Ender and Helgebostad, 1945; Lieck and Ågren, 1944
C. carpio	+	Chaet, 1950; Deutsch and Hasler, 1943; Lieck and Ågren, 1944; Woolley, 1941
Hybognathus nuchalis regius	+	Present work
Leuciscus erythrophthalmus	+	Lieck and Ågren, 1944
L. idus	+	Ender and Helgebostad, 1945; Lieck and Ågren, 1944
L. rutilus	+	Lieck and Ågren, 1944
Leucosomus corporalis	+	Present work
Notropis bifrenatus	+	Present work
N. c. cornutus	+	Present work
N. hudsonius amarus	+	Present work
N. v. volucellus	+	Present work
N. atherinoides	+	Deutsch and Hasler, 1943; Wolf, 1942
Pimephales p. promelas	+	Deutsch and Hasler, 1943
Rhinichthys a. atratulus	+	Present work
Semotilus a. atromaculatus	+	Chaet, 1950; Deutsch and Hasler, 1943
Tinca vulgaris	+	Lieck and Ågren, 1944
Family Centrarchidae:		
Ambloplites r. rupestris	−	Chaet, 1950; Deutsch and Hasler, 1943
Huro salmoides	−	Deutsch and Hasler, 1943
Lepomis auritus	−	Present work
L. gibbosus	−	Chaet, 1950; Deutsch and Hasler, 1943
L. m. macrochirus	−	Chaet, 1950; Deutsch and Hasler, 1943
Micropterus d. dolomieu	−	Deutsch and Hasler, 1943
M. salmoides	−	Present work
Pomoxis nigro-maculatus	−	Chaet, 1950; Deutsch and Hasler, 1943

2 of the family Centrarchidae lack thiaminase.

2. There is an indication that the distribution of the enzyme thiaminase is of some phylogenetic significance. The survey of forms which contain and those which do not contain thiaminase is as yet inadequate to explain the role which this enzyme might play in the distribution of animals.

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UTILIZATION OF PHOSPHORUS IN TROUT AS STUDIED BY INJECTION OF RADIOACTIVE PHOSPHORUS[1]

F. R. HAYES AND L. H. JODREY

Zoölogical Laboratory, Dalhousie University, Halifax, N.S., Canada

INTRODUCTION

RADIOACTIVE phosphorus has been the most extensively applied isotope from the discovery of artificial radioactivity to the present, because it excels in its rate of decay, quality of signals, freedom from danger, and ready availability. The distribution of work, however, has not been very widely spread over the animal kingdom. For instance, Hevesy (1948) in his recent monograph, gives 331 references to P^{32} experiments with named animals, which are grouped as follows: mice and rats, 161; man, 52; other mammals, e.g., dogs, cats, and rabbits, 83; hen, hen's egg, etc., 28;

[1] The authors wish to thank Dr. C. C. Coffin, who first suggested the use of tracers in this department and who made available to us the counting facilities of the chemistry department. Mr. D. A. Livingstone very kindly assisted in injecting the animals. Trout were made available from the rearing ponds at Grand Lake, N.S., through the courtesy of the Dominion Fish Cultural Branch. The investigation was supported by a grant from the National Research Council.

frog, 2; fish, 1; invertebrates, 4. The single fish paper is a brief account of the skeletal metabolism of the stickleback, *Gasterosteus* (Hevesy, 1945). Further information about phosphorus metabolism and turnover rate in lower vertebrates is evidently desirable, and to this end a study was carried out on trout during the winters of 1948 and 1949.

MATERIAL AND METHODS

Eastern brook trout, *Salvelinus fontinalis*, were used in the experiment, being secured from rearing ponds and maintained in aerated aquaria in a refrigerated laboratory at the university. They were yearlings, ranging in weight from 12 to 20 gm. To approximately one hundred of these trout the radioactive isotope, P^{32}, in the form of KH_2PO_4, was administered by intraperitoneal injection, and the animals were subsequently killed at intervals. Their tissues were then dissected and prepared for radioactive assay, as well as for total phosphorus determinations.

The P^{32} employed was obtained from the laboratories of the National Research Council. It was contained in crystals of KH_2PO_4 and was received in shipments of 0.1–0.7 millicurie. The weight of the phosphate was usually such that 1 gm. had an activity of 0.1 mc. In making up the solution used for injection, preliminary calculations were made in order to insure that about twelve thousand counts per minute (as detected on our counter and scaler) would be obtained from 1 ml. or less of solution. To the active salt dissolved in water enough sea water was added to bring the mixture to isotonicity with trout coelomic fluid. Thus it was presumed that injection did not interfere with the maintenance of osmotic balance in the fish. The solution was injected into the coelomic cavity with a micropipette fitted with a hypodermic needle. A micrometer attachment allowed the volume to be accurately controlled, measured, and kept constant. Depending on the activity of the solution, $\frac{1}{2}$–1 ml. was injected.

In an attempt to demonstrate a difference in P^{32} uptake due to temperature and dietary state, four different groups of trout were injected with phosphorus. Two groups were acclimated to 12° C., one of these groups was fed raw liver, and the other group fasted. A third group was kept at 2° C. and fed raw liver, while another group was maintained at 6° C. with no food given. The results from the groups did not vary sufficiently to permit conclusions to be drawn regarding the effect of temperature and diet on phosphorus uptake. It was therefore decided to average the results from the groups; and the values given in Figure 1 and 2 and in Tables 1, 2, and 3 are derived from all four series. In all cases the points on the curves are plotted as running averages, i.e., each point is the average of the original one, the point immediately before it, and the point immediately after it. This does not alter the form of the curve but tends to make it smoother.

The animals were killed at geometrically progressive intervals after injection, the earliest being 15 minutes and the latest 52 days. Portions of bone, liver, stomach, muscle, and heart were taken for analysis. The samples were weighed, digested in perchloric and nitric acids, treated with sulphate molybdate solution, and allowed to stand overnight while the phosphorus was precipitated as phosphomolybdate. For collection of the precipitate, disks of filter paper to fit the tray of a Geiger counter were cut out with a cork-borer and ringed with a wax pencil to prevent the precipitate from creeping over the edges. The disks were placed on a sintered glass filter, and the precipitate was collected by suction as

the suspension was dropped onto the disks from a medicine dropper. The size of the samples was kept constant, and in no case was the thickness of the precipitate great enough to require corrections for self-absorption.

Samples of water from the trout tanks were also taken at intervals and evaporated, in order to determine the amount of phosphorus which the trout returned to the water.

The radioactive samples were counted with a Geiger tube and scaler, the daily deviations of which were noted and corrected by counting a uranium standard. The background, chiefly due to cosmic radiation, was subtracted. Correction for decay was made by determining from a decay curve the percentage of P^{32} which remained at the time of counting and multiplying by the proper fraction. Corrections for counter deviation and decay may be eliminated by the use of a standard P^{32} sample made up at the same time as the injection solution. Such standards were made up as a routine and used alternately to the uranium standard as convenience dictated.

The corrected counts per minute, as obtained above from the samples, were brought to counts per gram by dividing by the sample weight. Since the activity of the solution injected into different groups of trout varied slightly from time to time, a standard activity of twelve thousand counts per minute was taken, and the tissue counts were multiplied by the appropriate factor in order to make the results comparable. Where counts are given as a percentage of the administered dose present in each gram of tissue, the counts were obtained as above, and this figure was expressed as a percentage of standard dose injected. Specific activity was calculated by determining the total phosphorus per gram of tissue and expressing the counts as a percentage of the administered P^{32} present per milligram of total phosphorus. Total phosphorus was determined by the colorimetric method of Robinson (1941), a photoelectric colorimeter being used.

Radioautographs were made of some trout and of some trout skeletons. The former were made by freezing the fish, sectioning sagittally, and placing the section on a high-speed dental X-ray film, protected from moisture by a thin layer of cellophane. Exposure times were determined by the activity of each sample, and the specimens were kept frozen during the exposure. In making radioautographs of skeletons, the fish was partially cooked in water until the vertebral column could be removed from the muscle. It was placed on film as before, but no refrigeration was necessary.

UPTAKE OF PHOSPHORUS

Figure 1 shows uptake of injected phosphorus by bone and liver. On the liver graph, a maximum may be seen at 2 hours after the injection. A maximum P^{32} concentration has been noted at about the same time in the liver of rats (Cohn and Greenberg, 1938; Perlman, Ruben, and Chaikoff, 1937) and was attributed by the latter authors to rapid phospholipid formation. A similar peak in potassium concentration appeared in the liver when radioactive potassium was injected into rabbits, which must have a different explanation (Fenn, Noonan, Mullins, and Haege, 1941). It appears possible that in the case of phosphorus, as well as potassium, the liver is taking on an excretory role by mobilizing the excess material injected. The decline is no doubt due to excretion (a considerable amount of P^{32} is liberated into the water at this time) and also due to replacement of active atoms by inactive ones. The gradual rise in P^{32} content of the liver probably represents an incorporation of phospho-

rus into various organic compounds, although no data were obtained on the activity of phosphorus fractions in this experiment.

It may be seen from the graph on bone uptake that the concentration of P^{32} per gram of bone is higher than that of liver. This is reasonable when it is considered that bone contains a great deal more phosphorus than liver tissue does. The organic fraction of bone in which the phosphorus atoms are not readily exchangeable (Cook, Scott, and Abelson, 1937; Manly and Bale, 1939). Bone phosphorus is relatively static but shows much more exchange when investigated with tracers than was previously thought (Hevesy, 1948, p. 409). There is some evidence, though not conclusive, to suggest that vitamin D acts to aid the con-

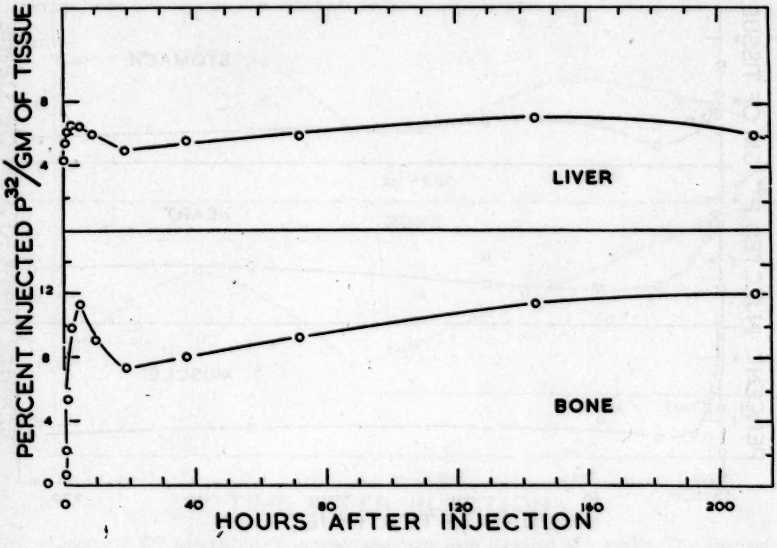

FIG. 1.—Uptake of P^{32} by liver and bone

maximum in P^{32} concentration occurring in bone at 4 hours is not very readily explained. It may be caused by some capacity of the apatite crystals to exchange phosphorus readily with the plasma, or it may be due to flooding of the extracellular spaces.

The decline in bone activity after the first peak is perhaps to be attributed to the fact that active atoms are becoming more scarce in the body (see Fig. 5 below). The later gradual rise presumably indicated a permanent holding of phosphorus. In mammals it has been shown that the P^{32} is chiefly deposited in the inversion of organic to inorganic phosphorus in bone (Cohn and Greenberg, 1939).

Figure 2 shows the concentration of P^{32} in stomach, heart, and muscle tissue. Stomach tissue shows the highest specific affinity of these three. Specific affinity is defined as the percentage of the administered dose present per gram of fresh weight. The highest concentration of P^{32} in the stomach is seen 15 minutes after the injection, which was the first observation made. The stomach was, of course, bathed by the injection solution in the coelom and might be expected to give high initial counts because of a me-

chanical holding of P^{32}. However, the liver, which was also bathed by the P^{32} solution, did not give highest counts immediately but rose to a maximum at 2 hours. It appears likely, then, that the counts recorded for both stomach and liver represent an actual uptake in some way by the organ in question. The maximum may be explained as a rapid dispersal of the added radiophosphorus into contained blood, for blood did not give any appreciable counts when assayed. Cohn and Greenberg (1938) have also found that blood in rats injected with P^{32} had little activity. In birds cardiac muscle has been shown to exhibit a greater phosphorus uptake than skeletal muscle (Entenman, Ruben, Perlman, Lorenz, and Chaikoff, 1938). This was also noted in the present work. The heart

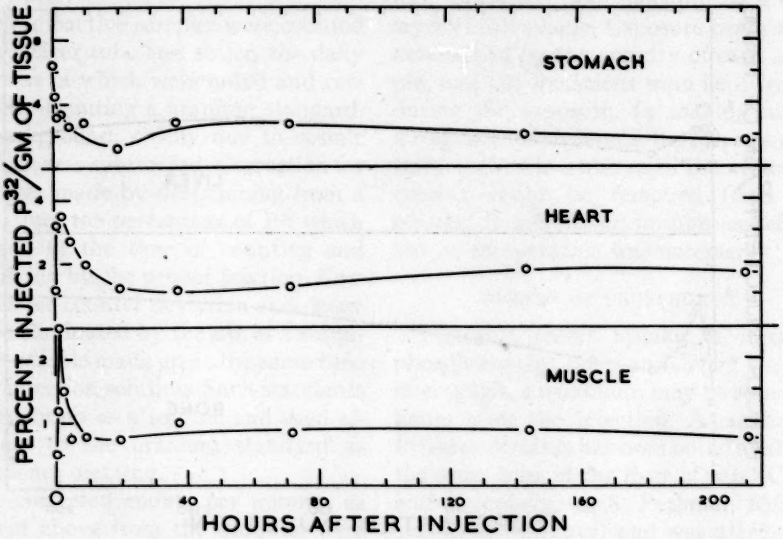

FIG. 2.—Uptake of P^{32} by stomach, heart, and muscle

the lymph spaces between the cells, while the subsequent decline would be caused by excretion of active atoms or their removal to muscle tissue. The latter, because its mass is very great, accounts for a large proportion of the P^{32}, even though its specific affinity is low. The slow gain in activity in stomach tissue could indicate the synthesis of organic phosphorus-containing molecules.

The heart, which was taken in its entirety because of its small mass, also reaches a peak in activity in the very short period of 1 hour after the injection. This is not due to a high activity in the exhibits the flooding shown by soft tissues. The decline may be due to causes mentioned for stomach and liver, and a later slight rise in P^{32} concentration suggests the synthesis of phosphorus-containing molecules.

Muscle shows the lowest specific affinity of all the tissues investigated. A sharp rise and maximum at 1 hour occurs, but the maximum is not so high as in the other tissues. This seems to support an assumption that the early maxima are due to filling of lymph spaces between the cells. Such spaces in muscle are small (see below), and therefore any

given weight of muscle would not be expected to contain as much active phosphorus as, for instance, liver. The decline may be due to excretion of active phosphate which took place to a large extent in the early stages of the experiment.

Inspection of Figures 1 and 2 shows that the levels of P^{32} reached after the flooding had declined (10–20 hours) were as follows: bone, 7; liver, 5; stomach, 2; at the end of 52 days. As P^{32} is not being excreted in appreciable quantities (see Fig. 5), the removal is probably to the skeleton.

A quite dissimilar curve is seen in the same figure for phosphorus retention in bone. Although there is considerable scattering of the points, it appears clear that the level of added phosphorus was still rising as the experiment closed.

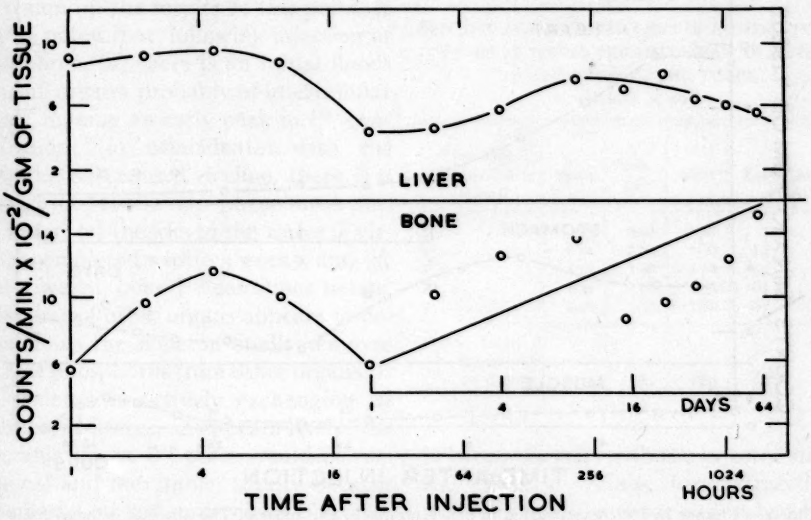

FIG. 3.—Change in P^{32} concentration in liver and bone over a period of 7 weeks. The abscissa is logarithmic.

heart, 1; muscle, 0.8. These represent the order of specific affinities of the various tissues for phosphorus.

Figure 3 shows results obtained from one group of trout investigated for a period of 7 weeks. Each point on these curves represents an observation from one animal alone and therefore shows more variation than did the previous curves. The curves are drawn with logarithmic abscissas, which distort the mental picture to a certain extent by exaggerating early hours and compressing later ones. In liver the rise seen in the previous graph appears to be followed by a decline There is evidence that phosphorus exchanges less rapidly in bone tissue than elsewhere (see Table 3, col. 5, below), so that when active atoms are incorporated into the inorganic portion of bone, they are not readily released again. Such phosphorus atoms would be taken up continuously at the expense of other tissues.

Figure 4 shows curves, from the same series of trout, for change in P^{32} content of heart, stomach, and muscle. The phosphorus content of the heart declines continually after the first maximum, evidencing a rapid turnover of initial phosphorus and lack of storage, in contrast to

bone. Stomach tissue activity declines after the first maximum, as shown in Figure 2, and shows a rise of doubtful validity in later phases of the experiment. Muscle continues to fall in P^{32} content for the first fortnight after injection. A maximum seen between the second and seventh weeks may be authentic or may be due to the fact that each point represents but one fish and individual variability enters.

Figure 5 represents the loss of P^{32} into the water, either from the site of the injection or by excretion. These data were obtained from the group of trout discussed in Figures 3 and 4. The loss shown here is cumulative, so that the final point represents the total loss of P^{32} from the

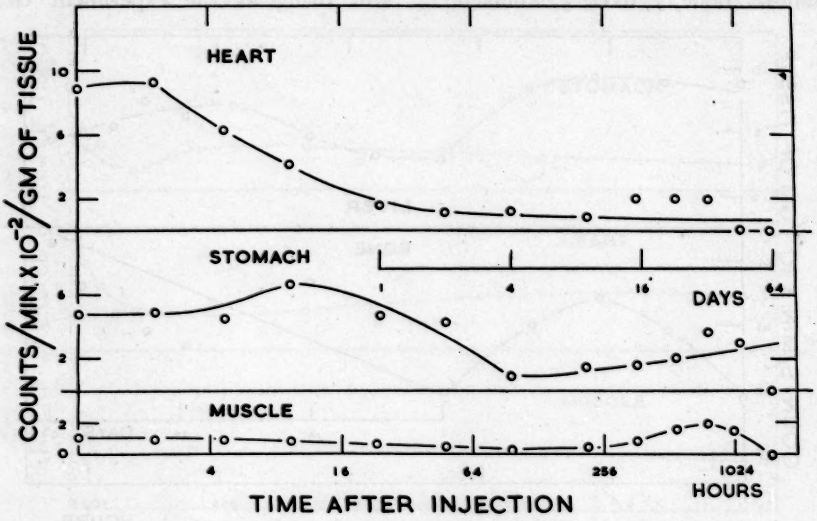

FIG. 4.—Change in P^{32} concentration in heart, stomach, and muscle. The abscissa is logarithmic

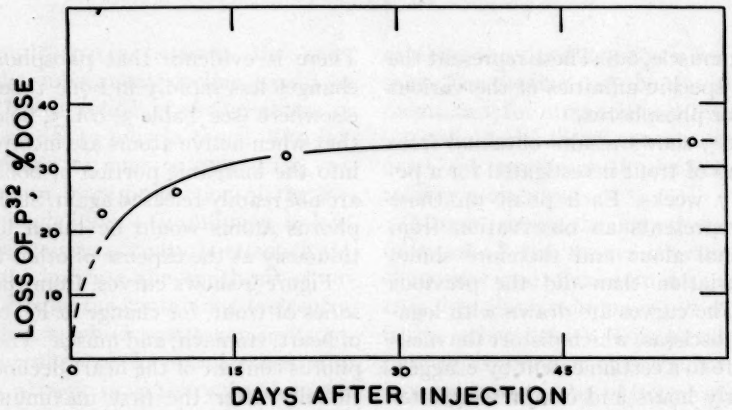

FIG. 5.—Curve representing the loss of P^{32} by the trout into the water. The initial loss might be from the site of the injection or by excretion.

animals at the end of the experiment. Since 34 per cent of the added P^{32} was accounted for in the water by the end of the experiment, it may be said that 66 per cent of the injected phosphorus was retained by the animals. The P^{32} was lost most rapidly in the early stages of the experiment, 23 per cent being present in the water when the first observation was made at the end of 3 days.

To sum up the results to this point, it may be noted that, following injection of phosphorus, (*a*) there is an initial flooding in all organs, probably of intercellular spaces, making an early peak in P^{32} concentration; (*b*) coincidental with the peak and subsequent decline, there is a marked loss of the new phosphorus into the water; (*c*) the loss to the water is virtually completed within 3 weeks; and (*d*) after 3 weeks, bone P^{32} continues to rise, while that of other organs appears to decline. Thus the skeleton finally removes the new phosphorus from other organs. If the skeleton is actively exchanging its phosphorus atoms, as appears likely, the accumulation of P^{32} there would be mechanical and inevitable, simply because bone is the largest reservoir of phosphorus. Similarly, if one dispersed a supply of new bank notes at random through a town, they would soon be found accumulating at the banks, even though the ratio of money in the banks to money outside remained unchanged.

SPECIFIC ACTIVITY AND RELATIVE TURNOVER RATE

In this paper we have expressed specific activity as the percentage of the administered P^{32} present per milligram of total phosphorus in each tissue. As seen in Table 1, the early values are irregular and reflect the peaks in Figures 1 and 2. After the postflooding stabilization, no clear trend is apparent, and the averaged results, from 9 hours on, show that specific activity decreases in the following order: liver, heart, stomach, bone, muscle.

Although early peaks, as in Figures 1 and 2, have been noted by other workers, as cited above, there appears to have been little attention paid to their cause. It is reasonable to suppose that flooding represents the filling-up of fluid spaces between the cells with the injected material. Precise values for extracellular spaces in fish tissues are not at hand but may be expected to parallel, in a general way, those of mammals. If we set down the maximum specific activity observed, as taken from Table 1, together with corresponding mammalian extracellular spaces, it turns out that the sequence of the two sets of values is the same, as shown in Table 2.

TABLE 1

SPECIFIC ACTIVITY: PER CENT OF ADMINISTERED P^{32} TO BE FOUND PER MILLIGRAM OF TOTAL PHOSPHORUS IN THE TISSUE
(Values × 10^2)

Time after Injection (Hours)	Heart	Stomach	Liver	Bone	Muscle
¼	99	240	130	4	20
½	260	170	160	14	45
1	270	120	170	35	96
2	290	130	190	64	59
4	240	99	180	67	28
Av. from 9 hours to 20 days	137	82	169	68	35

A calculation of the relative rate of phosphorus turnover in the tissues may now be attempted. For this some assumptions are necessary:

a) There was no appreciable growth of the trout, so that material was being removed at the same rate as it was formed, i.e., for every atom of new phosphorus going in, there was an atom of old phosphorus going out.

b) The same or a known amount of P^{32} was present in the plasma at the beginning and end of the interval considered.

c) Temporary addition of P^{32} to tissues by flooding at the start should not be counted as metabolic turnover.

d) All exchange by the cells was in one direction, namely, uptake from zero at the start to a certain level at the end of an interval. In other words, the loss of P^{32} by the cells is taken as negligible. Later on, of course, P^{32} will be turning over like the rest; when this occurs, radiophosphorus no longer serves for the measurement of rate of turnover.

TABLE 2

COMPARISON OF THE MAXIMUM SPECIFIC ACTIVITY OBSERVED IN FISH AND THE CORRESPONDING EXTRACELLULAR SPACES IN MAMMALS

Tissue	Specific Activity × 10 (Trout)	Per Cent Extracellular Space (Mammal)*
Heart	29.0	30
Stomach	24.0	30
Liver	19.0	23
Muscle	9.6	11
Bone	6.7	.....

* Mammalian data from Manery and Bale, 1941.

Inspection of Figures 1 and 2 shows that after the initial peak there is a fairly well-defined minimum, occurring in most cases at about 24 hours. During this first 24 hours one might think of the added P^{32} as providing a constant head of pressure on the cells, the initial excess being backed up in flooded areas until, by excretion, the system dropped to equilibrium. Figure 5 shows how the excess is drained off rapidly in early stages. After the trough, the curves on Figures 1 and 2 tend to level off. When they are completely level, it means that P^{32} is leaving at the same rate it is added, and no calculation of turnover is possible from our data. One may, however, calculate specific activity from the trough point at which equilibrium was reached after flooding and before the tissue losses of P^{32} began to balance out the gains.

Because of a deficiency in observations of plasma activity, our data do not permit determination of absolute turnover rate. However, from the relative rates of change of specific activity in the various tissues, relative turnover rates can be calculated, and the tissue whose phosphorus is being renewed most rapidly can be determined.

It is seen from column 5 of Table 3 that very considerable differences exist in turnover rate. This is not surprising: for instance, the stomach, with a very rapid rate, has actually the function of passing material through its cells; on the other hand, bone, with the lowest rate, is a residual storage site for phosphorus.

TABLE 3*

TURNOVER RATES FOR VARIOUS TISSUES

Tissue	Selected Time Interval (Hours)	Specific Activity	Calculated Specific Activity at End of 1 Hour	Relative Turnover Rate
(1)	(2)	(3)	(4)	(5)
Liver	18	1.40	0.08	32
Bone	18	0.47	0.03	12
Muscle	4	0.28	0.07	28
Heart	18	0.98	0.05	20
Stomach	4	1.00	0.25	100

* Column 2 is the time of the first observation after the flooding of the tissue was over. Column 3 values are obtained as described for Table 1. Column 4 is column 3 divided by column 2. Column 5 is column 4 adjusted to whole numbers, with maximum taken as 100.

RADIOAUTOGRAPHS

The radioautograph of Figure 6 shows considerable concentration of P^{32} in the skeleton, including the vertebral column, bases of fins, jaws, and skull. Some P^{32} is

also present in the viscera. This fish was killed 9 days after the injection.

Figure 7 is a radioautograph of part of the vertebral column and tail of a trout killed 4 days after the injection. P^{32} is very evident in the bone.

Figure 8 is a radioautograph similar to Figure 7 but made from a fish killed 6 days after the injection. Both radioautographs were given the same exposure time (17 hours), but the one shown in Figure 8 is considerably darker, showing a greater concentration of P^{32}. This is in agreement with the graph on P^{32} uptake by bone.

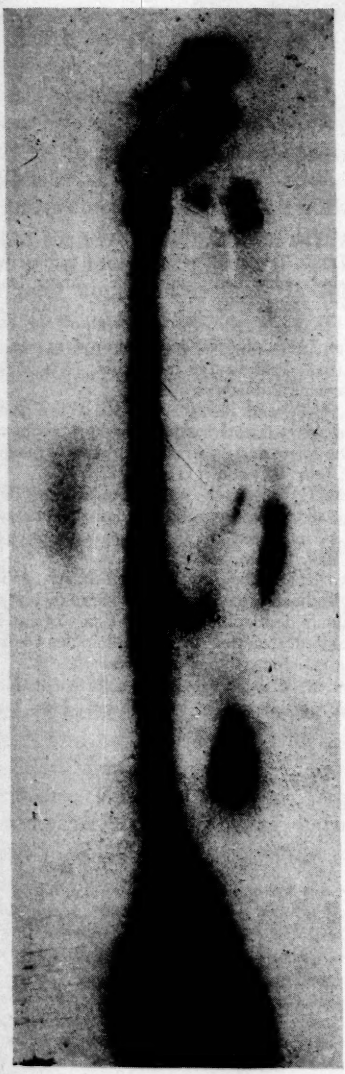

FIG. 6.—Radioautograph of a trout killed 9 days after injection. Exposure 21 hours

In a study of uptake of P^{32} by fish in a lake, the added material was detected by radioautographs in the viscera after 9 days and in the skeleton after 15 days (Coffin, Hayes, Jodrey, and Whiteway, 1949). Counts indicated that the maximum concentration occurred after 3 weeks of exposure.

SUMMARY

1. Approximately one hundred yearling trout (*S. fontinalis*) of 12–20 gm. were injected intraperitoneally with about twelve thousand counts per minute of radiophosphorus, and tissue counts were made at intervals thereafter.

FIG. 7.—Posterior part of vertebral column of trout killed 4 days after injection. Exposure 19 hours.

2. Peaks in P^{32} concentration appeared in all tissues, usually within 1–2 hours after injection, and were attributed to flooding of the spaces between the cells with the newly added phosphorus and, in the case of the liver, to an excretory process.

3. After the peak, when trends had become stabilized, the specific affinities (per cent dose per gram of organ weight)

were in the following order: bone (highest), liver, stomach, heart, muscle.

4. The rate of turnover of phosphorus was most rapid in stomach tissue. Rates in the other tissues were considerably lower and decreased in this order: liver, muscle, heart, bone.

5. At the end of 52 days, the trout still retained 66 per cent of the initial dose.

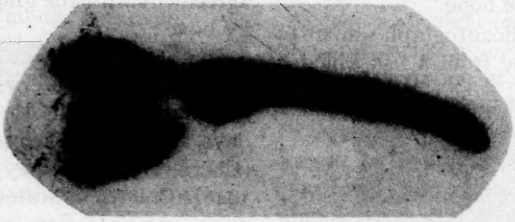

FIG. 8.—Vertebral column of trout killed 6 days after injection. Exposure 17 hours

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THE EFFECTS OF BIOTIN DEFICIENCY ON HAIR DEVELOPMENT AND PIGMENTATION[1, 2]

HAROLD RAUCH
Department of Zoölogy, University of Massachusetts, Amherst

In a recent paper Chase, Rauch, and Smith (1951) describe the normal development of the hair in the mouse. Using the terms originally suggested by Dry (1926), the developmental cycle is divided into three phases. The anagen or active phase is characterized by the proliferation of the hair germ, the formation of the bulb, and the production of the hair. Since this is the phase of the most abundant changes in the follicle, it is further divided into six substages, anagens I–VI (Chase *et al.*, 1951). The catagen phase is distinguished by the cessation of mitotic activity in the bulb, its inversion, and the formation of the club. The telogen, characterized by the presence of the club hair, is the resting or inactive stage. With this description of the normal development as a starting point, a variety of experimental techniques have been employed. In this paper the effects of biotin deficiency on hair development are reported.

The biotin deficiency syndrome in the rat is described by Sullivan and Nicholls (1942) and by Emerson and Keresztesy (1942); a similar syndrome is found in the mouse (Wilson *et al.*, 1949). Of prime concern here are the effects of the deficiency which pertain directly to the hair. Biotin deficiency is considered, then, in relation to the development, the retention, and the pigmentation of the hair.

MATERIAL AND METHODS

The mice used were of the C57 Black strain from the colony of Herman B. Chase of Brown University. For the production of the deficiency, fifty-two animals, 16–19 days of age, were weaned on the diet described by Wilson *et al.* (1949), in which the effective factor is egg white.[3] In order to eliminate biotin production by intestinal flora, this diet was supplemented with 0.5 per cent sulfaguanidine (Black *et al.*, 1941). The deficiency thus produced has been attributed to an egg-white injury (du Vigneaud *et al.*, 1940).

Since the plucking of club hairs has been shown by Collins (1918) and by David (1934) to initiate the growth of the succeeding hair generation, this technique was employed to obtain all developmental stages. During the 5 weeks up to the time of marked deficiency, regions of the back were plucked at intervals, and gross observations on hair regeneration were made. After this time, minimal doses of biotin, approximately 2 μg., were given orally while the animals were continued on the diet. Tissues were taken for examination during the period of marked deficiency.

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[2] This work was supported in part by a grant-in-aid to Dr. Chase from the American Cancer Society, recommended by the Committee on Growth of the National Research Council. I am grateful to Dr. Herman B. Chase, who supervised this research and was always ready with advice and encouragement.

[3] I would like to thank Henningsen Lamesa, Inc., for the egg white; Merck and Company, Inc., for the biotin; Lederle Laboratory Division, American Cyanamide Company, for the sulfaguanidine.

RESULTS

The diet used is effective in the production of marked deficiency after 6 weeks, at which time appreciable alopecia and achromotrichia are observed. Prior to this, less pronounced but recognizable symptoms are observed.

ACHROMOTRICHIA

Within 2 or 3 weeks the coat loses its luster, taking on a faded, dull appearance. This is due not to a change in pigmentation but rather to the lack of sebum on the surface of the hairs (Montagna, 1950). During the following weeks, however, a true decoloration develops. The first appearance of the decoloration is during the third week, when in various regions reddish or gray hairs are observed. In the fourth week the hairs of other regions become red or gray, but these are consistently more lightly pigmented than were the first regions to become decolorized. During the fifth and sixth weeks there is further lightening of the coat, so that former red regions become yellow and gray regions become silver. The achromotrichia is generally completed by the end of the sixth week with the appearance of yellow or silver hairs. It is found here that the direction of the decoloration is dependent on the age at which the animals are weaned on the diet. Thus in animals weaned early, at 16 days of age, the decoloration proceeds from black through gray to silver, whereas in those weaned later, at 19 days, the change is from black through red to yellow.

Examination of individual hairs taken during the fourth and fifth weeks reveals a progressive lightening of pigmentation along the shafts, so that the tips are darker than the bases. When the animals are plucked during the first 4 weeks after the introduction of the diet, the new hairs appearing in the plucked regions are consistently lighter than the hairs of the surrounding unplucked regions. Thus the next hair generation advances a stage in the decoloration sequence. It is apparent, then, that the shaft, once formed and pigmented, does not change its color; instead, the lightening of the coat is due to the replacement of dark hairs by lighter ones, so that there is a loss of color from hair generation to hair generation.

If animals which have been on the diet for several months and are extremely deficient are given single doses of 2 μg. of biotin, there is striking evidence of a partial alleviation of the symptoms within 24 hours. A sudden and pronounced darkening of the skin appears in several areas on the back. These areas are generally bald or at the most have very short hairs. The dark skin persists for about 10 days, during which the hairs emerge through the skin and increase in length. All the hairs in these regions exhibit light tips and dark bases at the completion of development, although the extent of the light portion is greater in those hairs which attain full length earlier. On the second, third, and succeeding days after the administration of biotin, other areas of the skin also darken. The earlier hairs completing development in these areas may be wholly black, while those developing later are black distally and light proximally.

In several animals the darkening of the skin proceeded in a wave along the back after treatment with biotin. The most anterior hairs to emerge were entirely light. These were followed in progression posteriorly by hairs showing light tips and dark bases, completely black hairs, and hairs with black tips and light bases. Finally, the most posterior hairs were all light.

ALOPECIA

The first appearance of alopecia occurs during the third week after the introduc-

tion of the diet and is generally on the venter of the neck. Beyond this there is apparently no specific pattern of hair loss. The epilation may proceed from the neck posteriorly over the back or begin anew about the tail or hind limbs and proceed anteriorly. The alopecia is gradual, so that an area appears first thinly covered, then sparsely covered, and finally, by the end of the sixth week, more or less bald. Occasionally small clumps or tufts of hair are scattered over an otherwise fairly bald back. These patches are not constant, for even during the period of marked deficiency these tufts fall out and others appear elsewhere. In some cases a narrow band of hairs extends transversely across the back. This band also is not constant, but seems to move down the back. This can be viewed as a normal wave of growth, in which the hairs, having reached approximately their full length, are shed, thus giving the appearance of an advancing line or of a sporadic patching. In each of these instances the hairs develop at a normal rate.

The animals are plucked during the time of early symptomatic deficiency prior to extensive alopecia. Pluckings during the first 4 weeks yield new hair generations which are completely normal with respect to rate and structural attainment. Areas plucked during the fifth week occasionally exhibit new hair growths, but more often remain nude. Where the latter is true, these areas frequently become the sites of the initial baldness. Later, at the time of appreciable hair loss, plucking is entirely ineffectual in initiating a new hair generation, despite the fact that new growths continue to appear normally.

If extremely deficient animals are given 2 μg. of biotin, the hairs continue to develop normally. Upon attaining approximately full length, however, the hairs are lost.

Histological examination of skin taken from bald areas reveals follicles which are in early stages of anagen or in telogen. The follicles in the anagen phase appear normal except for the absence of black pigment in the bulbs and hair shafts. Although clubs are occasionally lacking, most of the follicles in the telogen phase exhibit normal clubs and club sacs. In these the shafts are broken just distal to the club, resembling in appearance normal follicles immediately after plucking.

DISCUSSION

The observations indicate that hair development occurs during biotin deficiency. The initial wave of growth is ordinarily completed by the time the special diet is introduced. The second wave commences shortly and is usually completed before most of the deficiency symptoms appear. The third hair generation develops normally as a fairly regular wave, despite other evidences of pronounced deficiency. In the normal mouse, later hair growths become less regular, so that by the fifth or sixth hair generations the hairs appear in patches. The same type of irregularity occurs in deficient animals. The rate of elongation of the hair shaft and the duration of the telogen phase in deficient mice coincide with those observed in animals receiving adequate rations.

Plucking of hairs of the first and second generations results in the initiation of development of the next hair generation, which proceeds normally. Plucking of hairs of the third or later generations, that is, during extreme deficiency, is ineffective in initiating additional hair growths. It is evident, then, that biotin deficiency diminishes in some way the receptivity of the hair follicle to the plucking stimulus, without affecting the

receptivity to the normal developmental stimulus. Thus biotin deficiency does not influence the rate and success of hair development, nor does it affect the duration of the intervals between developments. It does, however, make the follicle unreceptive to the plucking stimulus.

The progressive alopecia, symptomatic of biotin deficiency, indicates a fault in the mechanism of hair retention. This fault lies probably in the incomplete keratinization of the proximal portion of the hair shaft. This is indicated by hair shafts broken just distal to the clubs in deficient animals. This suggests that lack of biotin is effective during the very end of anagen and the catagen or club-formation phase of development.

A pattern of alopecia for the dorsum cannot be prescribed. It should be recognized that variation in the amount of stored biotin and thus in the time of the onset of the deficiency exists among individual mice. Since it has been shown that the deficiency is effective only at a particular stage of the developmental cycle, it must be expected that only those hairs which are in late anagen and catagen at the time of the reduction of the biotin content below the minimum level will be immediately affected. These areas are the sites of the initial alopecia. In other areas there is a delay until the hairs reach the appropriate stage, at which time the response is apparent. This explains the variation in the sites of the initial alopecia and also its spread. Since development at this time generally proceeds in a wave, it is to be anticipated that the critical stage is attained progressively in adjoining areas, so that hair loss also advances in a wave. It has been reported that alopecia is frequently symmetrical. This is not surprising in view of the fact that in young animals the waves of growth are for the most part regular and symmetrical.

The rapid appearance of hairs in denuded areas after continued injection of biotin has been reported with the suggestion that this supplementation is responsible for the new growth. No curative action of biotin in this respect is possible, since the deficiency does not interrupt the normal developmental cycles. The new hairs are rather those which would have appeared had no biotin been administered. The real effect is that these hairs are retained when fully elongated, since by that time the level of biotin has been raised sufficiently to allow normal shaft and club formation. In the present study it appears that the administration of single small doses of biotin at early and late anagen and catagen stages raises the level slightly, but not enough to prevent completely the defective keratinization. Continued administration of biotin would allow the retention of a normal coat.

Administration of a single small dose of biotin has a striking effect on color. Within 24 hours, there is a darkening of the skin in regions in which the follicles are in anagens IV–VI. This darkening of the skin is due to the appearance of black pigment in the hair shafts and bulbs below the surface of the skin. The first hairs to emerge from this region are light at the tips and dark at the bases. These hairs, evidently well into anagen VI at the time of administration, have lightly pigmented portions of the shaft already formed. Farther along the wave, hairs appear which are entirely black. These hairs were at the stage of the beginning of normal pigment production (anagen IV) at treatment, and thus are wholly pigmented. Still further along the wave are hairs which are dark only at the tips. These hairs were not yet ready for pig-

mentation (anagen I–III) when biotin was given. Since the biotin level is maintained for only 10 days, the distal portions of the shaft will be dark, while the proximal portions, developing after the 10 days, will be light. Finally, only light hairs are found. These hairs were not ready for pigmentation until the biotin supply was again depleted. These observations suggest that biotin affects a late step in the pigmentation process and that this process is more sensitive to changes in the biotin level than is shaft development.

SUMMARY

The symptoms of biotin deficiency produced by a diet containing egg white include alopecia and achromotrichia. During deficiency the normal cycles of development are maintained, although the hairs are shed at the completion of growth, indicating imperfect keratinization of the proximal portion of the hair shaft. It is concluded that biotin is necessary neither for hair development nor for the normal initiation of hair development. Since plucking is ineffective in inducing the development of the next hair generation during deficiency, it is suggested that biotin is involved in the response to the plucking stimulus.

Single doses of 2 μg. of biotin are effective, at certain stages of hair development, in restoring normal pigmentation within 24 hours. Therefore, it is apparent that biotin is required for a final step in the pigmentation process, which is particularly sensitive to changes in the biotin level.

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SPUR GROWTH AND DIFFERENTIATION IN THE ADULT THIOURACIL-TREATED FOWL[1, 2, 3]

MARY JUHN

Department of Poultry Husbandry, University of Maryland, College Park, Md.

IT HAS been shown by Astwood, Bissel, and Hughes (1944) that thiouracil via its interruption of the secretory activity of the thyroid will cause a marked retardation of growth in the young chick. When thiouracil was fed as 0.5 per cent from within the first week of age onward, the muscles became weak and the joints became hypermobile. The head furnishings and the spurs failed to develop, and the down of the body was not replaced by the juvenile plumage as is normal. The authors noted, though, the growth of a "fringe of wing feathers." By this is meant probably the distal sections of certain of the primaries and secondaries, which, however, are laid down prior to hatching. Accordingly, the drug would appear to be less effective in interrupting growth already well under way when first given.

In the mature fowl, thiouracil profoundly influences the development of newly forming feather sections (Juhn, 1944*a;* Domm and Blivaiss, 1944). New feathers are available in the later life of the bird because of the persistent regenerative capacity of the plumage, which, because of this and other properties, has frequently been compared to an embryonic system. Aside from the plumage, definite modifications are obtained in the spur of adult male fowl (Juhn, 1946, 1948). These are similar to modifications described earlier by Caridroit and Régnier (1944) following thyroidectomy of adult fowl and involve primarily the locus of attachment to the tarso-metatarsus but also may lead to alterations of the shape of the spur proper.

This continued malleability naturally suggests the presence of developmental peculiarities which set the adult spur apart from the other bony structures. The present, more extended investigations on the action of thiouracil on the spur of mature fowl were directed, therefore, first toward an examination of the normal processes of calcification, with particular reference to their time of onset. Studied also were the respective roles of the age of the fowl and the stage

[1] The developments discussed in this paper are based entirely upon records furnished by X-ray films of the spur region of normal and experimental birds. These photographs were all taken by Dr. J. F. McClure, of Hyattsville, Maryland, for whose continued interest and collaboration I wish to express my especial appreciation. I also wish to thank Mr. John Spurbeck, Johns Hopkins University, who prepared the plates accompanying this paper, and those students of the Poultry Department of the University of Maryland who at one time or another assisted me in the course of this work.

I am particularly indebted to Dr. George F. Briggs, then of this department, who suggested the dietary supplements used, and to Professor George D. Quigley, University of Maryland, for the preparation of the capons and slips used in certain of the tests.

My thanks are due to Dr. Mark Welsh, Lederle Laboratories, Pearl River, New Jersey, for the thiouracil and to Dr. Erwin Schwenk, Schering Corporation, Bloomfield, New Jersey, and Dr. F. E. Houghton, Ciba Pharmaceutical Products, Summit, New Jersey, for the crystalline testosterone. Dr. Robert L. Kroc, Chilcott Laboratories, Morris Plains, New Jersey, very kindly furnished the relaxin preparation.

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[3] This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

of development of the spur at the commencement of thiouracil treatment in the ensuing modifications.

A number of dietary supplements and three hormones—thyroxine, testosterone, and theelin—were given jointly with thiouracil for possible augmentation or nullification of the drug-caused lesions. All experimental fowl were kept under observation after cessation of a given treatment, for possible repair in the spur as survival permitted. As an extension of these investigations, records of spur condition were made in normal males injected over protracted periods with theelin and with stilbestrol alone. The possible properties of relaxin in modifying the internal spur-shank junction were similarly followed. The effects of thiouracil were followed also in the spurred hen, and comparisons of the action of the drug were made between cock, slip, and capon.

PROCEDURE

The birds serving in this study were raised at the University of Maryland farm. The fowl all belonged to the heavier breeds, being for the most part New Hampshires, cross-breds from White Plymouth Rock ♂—New Hampshire ♀ matings, and some few Barred Rocks. They were brought to the laboratory as adults and were housed in individual adjacent cages. Since it is well known that confinement in batteries often leads to an overgrowth of the head furnishings, which renders proper nutrition difficult, the males were dubbed at that time. Feed and water were continually available. Records of body weight were made once a month.

Thiouracil was fed in all cases as $\frac{1}{2}$ of 1 per cent of the standard dry mash used. In adults this level proved relatively nontoxic, fowl survived for periods of years, and aggressive individuals retained this behavior pattern. However, special care was essential in handling the birds. As the well-known thyroid hyperplasia developing in response to the drug set in (Astwood, Bissel, and Hughes, 1944), any—even slight—pressure in the region of the glands led to death, apparently by suffocation. An example of the extreme cystic enlargements encountered is shown in Plate I, where photographs of the right and left thyroids from a treated cock are reproduced in comparison with the normal.

The thiouracil-treated fowl early commenced a continuous shedding and replacement in sporadic sections of the plumage; the new feathers promptly showed the characteristic structural modifications—an elongation of the vane and a diminution of barbulation. Each experimental bird was regularly inspected for developing feathers in order to check on the continued effectiveness of the treatment, since the reappearance of the normal pattern would indicate the development of a refractory state.

X-ray photographs were always taken prior to any tests, and fowl were assigned to experimental groups only after a preliminary study of these pictures. The spur was held in the same position at all exposures so far as possible, and the foot and hock were included in the photographs for reference. The films were placed for immediate examination on a ground-glass viewer, and all the measurements recorded were made directly from them. In order to obtain comparable estimates of the length of the spur and comparable definitions of the degree of its internal calcification, a point was first located on the tarso-metatarsal surface at the center of the section of spur attachment. The distance in a straight line from this point, o, to the tip of the spur was taken as spur length. The position of the first indications and the later proximal-distal limits of the calcified zone

were located on this same axis. Old spurs tend to show curvatures, notably of the distal segments; in these circumstances the lengths obtained are approximations. The calcified sections, however, usually do not extend to those distances.

young and older adults, of good weight and condition, and all the spurs were externally well-developed, firm structures.

It will be seen from Table 1 (see also Pl. II) that first spur calcification occurs late and that the process follows definite

TABLE 1

SPUR AND CORE LENGTHS AND NORMAL STAGES OF SPUR DIFFERENTIATION IN THE ADULT COCK

Bird No.	Age (Months)	Breed	*D** (Mm.)	Core (Mm.)	Spur (Mm.)	Socket
724	7	Cross	.....	.....	9	.....
714	9	NH	1	3	9	.....
737	9	NH	.....	.....	11	.....
719	7	Cross	2	6	11	.....
735	7	Cross	4	1	12	.....
734	7	Cross	4	4	12	.....
736	9	Cross	3	6	12	.....
758	8	BPR	.....	.....	13	.....
721	9	Cross	2	8	13	.....
722	9	Cross	2	9	13	.....
717	9	NH	1	10	14	.....
715	7	Cross	0	10	14	.....
755	8	BPR	6	2†	15	.....
731	9	Cross	5	8	15	.....
725	7	Cross	3	8	15	.....
730	7	Cross	4	8	16	.....
750	9	Cross	4	10	17	.....
720	7	Cross	2	11†	17	.....
740	7	Cross	4	10	18	.....
760	8	Cross	4	11	18	.....
749	9	NH	3	11	19	.....
712	7	NH	0	17	19	.....
752	7	NH	0	17	19	.....
702	18	WPR	0	26	37	Present
703	18	NH	0	31	42	Present
706	30	BPR	0	31	43	Present
705	18	NH	0	33	44	Present
704	18	NH	0	35	51	Present

* *D* is the distance separating the core of the spur from the tarso-metatarsus.
† Fragments.

PROGRESS OF NORMAL CALCIFICATION

The normal progress of spur calcification could be read in a series of stages that were found represented among a large group of untreated cocks at the time of a preliminary inspection. This general picture was supplemented in detail by continuous records made in certain individual birds. The successive stages of spur calcification are described in Table 1. The birds are listed in order of increasing spur length. The cocks were

spatial directions. No calcification is present in three individuals (♂♂724, 737, 758). Minute granules are visible at a distance of 4 mm. from the tarso-metatarsal surface in ♂ 735, and fragments are present in ♂ 755. The next stages show the organization of a diminutive central core. The spur proper is gradually increasing in length with these developments, and for a considerable period the distal apex of the central core tends to retain its relative position to-

ward the spur tip while progressively extending its proximal margin toward the tarso-metatarsal surface. Next, at a certain point of its advance toward the shank, the core throws forward slight dorsal and ventral swellings. Simultaneously, the hitherto smooth surface of the shank gives rise to two small thickenings that project toward these paired lobes. The tarso-metatarsal proliferations increase in size and expand to fuse with each other while leaving the center free, thus forming a ring-shaped structure—the socket primordium. At the same time a delicate series of fibers arise, connecting this primordium with the adverted core surface. These fibers are the scaffolding, so to speak, for the final developments, in which the socket wholly embraces the basal section of the spur core. Calcification of the socket is, in general, most dense at first in its distal segments, but there follows a proximally directed equalization. With increasing age of the cock, growth in the length of the core lags in the face of the continued growth in the length of the spur, and so it comes to occupy an ever smaller segment of the organ (see, e.g., ♂ 704, Table 1). Additional numbers of much more pronounced cases have been recorded in males several years of age, in which spur length often becomes extreme.

EXPERIMENTAL

EFFECTS OF THIOURACIL ON SPUR CALCIFICATION

Since the processes of calcification in the spur first arise so late in the life of the fowl and then continue so gradually, it is not possible strictly to dissociate differences of response due to age from those having an explanation in the developmental stage initially present. A suitable approach in comparing older with younger fowl consisted in selecting all for completed junction of the core with the tarso-metatarsal surface. Four cocks, 18 months of age, and four cocks, 9 months of age, were continued under identical experimental conditions. Thiouracil was fed for 474 days and regular mash for 326 days longer. The surviving birds were then autopsied.

The two treated age groups proved similar, in that both the spur and the internal core continued to increase in length at a fairly steady pace for a very

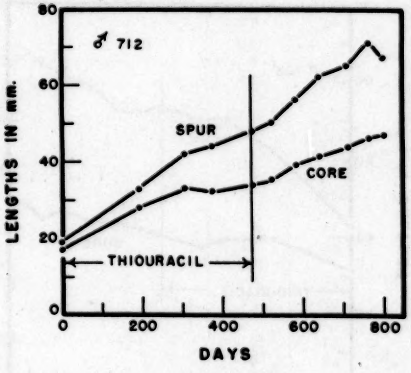

FIG. 29.—Growth in lengths of spur and core during and following thiouracil treatment; ♂ 712, 9 months of age at beginning of test.

considerable period, although more rapidly in the younger fowl. After about 300 days of the thiouracil diet, however, spur growth slowed, and core size became slightly reduced in all cases. After an interval following cessation of treatment, both spur and core growth were gradually resumed, somewhat more slowly in the older group.

The changing linear dimensions of the core and spur during thiouracil treatment and after its interruption are shown graphically in Figure 29 for a member of the younger, and in Figure 30 for a member of the older, group of birds.

The actual internal structure of the spurs in the two age groups was, however, very differently affected. The older birds showed practically no changes be-

yond a very slight decalcification of the core apex and occasional, almost imperceptible, indentations of the socket surface. These defects appeared with the gradual decline of growth and became slowly eradicated with its renewal. In all the younger group spur organization early took an abnormal course. Actual full development of the socket to reinforce the connection of the core with the tarso-metatarsus was not completed; the

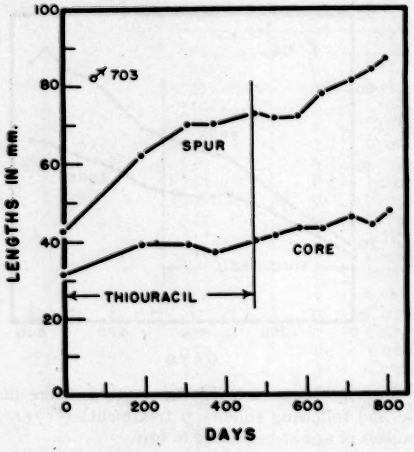

FIG. 30.—Growth in lengths of spur and core during and following thiouracil treatment; ♂ 703, 18 months of age at beginning of test.

union was tenuous. The core proper became of abnormal shape, with lobulated margins and a weakly calcified apex. In one case the entire distal segment of the core was sloughed off, much like a pulp cap in a feather; another exhibited the preparatory stages. With the cessation of thiouracil treatment, repair set in slowly, but there was no true return to normal structure. Socket-like formations, for example, gradually arose, but these were spreading, diffuse masses instead of having the characteristic sharply limited outlines. It is clear, therefore, that age levels, even in adult fowl, are of very considerable importance to the degree of thiouracil response in the spur.

In an investigation of the opposite situation, spurs of initially differing development were systematically followed in cocks of closely comparable age. Two groups of ten birds each were selected to parallel each other so that every step represented in the one group was also found in the other. The series of stages ranged from total lack of spur calcification to core-shank junction. The cocks were also similarly distributed by age between the group to receive thiouracil and the other to serve as control.

The measurements recorded in this series of observations are entered in Table 2. Comparisons are made between treated and control cocks paired on a basis of similarity in initial degree of spur differentiation. The original condition of the spur is entered and its state 125 days later, when these tests had been completed. An examination of Table 2 will show clearly that a critical limit in the distance separating the core from the tarso-metatarsus determines the reaction to thiouracil. When thiouracil administration is commenced before the core approaches this boundary, its union with the shank will not take place. If, however, the core has already extended beyond this line prior to treatment with the drug, the junction will be effected. This is in the absence of actual physical contact, as may be judged from the X-ray photographs, and in face of the later abnormalities which come to accompany the long-term use of thiouracil in the male fowl.

Other fowl, similarly paired with reference to initial similarity in internal spur development and identity of age and breeding, were continued for prolonged periods of observation. In Table 3 (see also Pl. III) are entered the data for one representative pair from this series. These two cocks were from one day's hatch of a White Plymouth Rock ♂–New Hampshire ♀ mating; normal spur

TABLE 2*

EFFECTS OF THIOURACIL AT SEPARATE STAGES OF SPUR DEVELOPMENT

Treated				Control			
Bird No.	*D*† (Mm.)	Core (Mm.)	Spur (Mm.)	Bird No.	*D*† (Mm.)	Core (Mm.)	Spur (Mm.)
724			9	737			11
	4	11	18		1	17	23
758‡			13	735			12
					0	24	27
731	5	8	15	755	6	6	15
	5	23	31		4	15	23
730‡	4	8	16	726	4	7	14
					0	27	30
740	4	10	18	734	4	4	12
	5	18	31		0	24	27
725	3	8	15	750	4	10	17
	0	20	28		0	28	34
736	3	6	12	761	4	9	18
	0	16	21		0	26	32
749	3	11	19	719	2	6	11
	0	30	37		0	21	24
720	2	11	17	721	2	8	13
	0	27	31		0	25	32
717	1	10	14	722	2	9	13
	0	23	29		0	19	24

* Test and control cocks are paired with reference to lack of calcification or to similarity in value for *D*. For each bird, upper line, initial stage; lower line, 125 days later.

† *D* is the distance separating the core from the tarso-metatarsus.

‡ Did not survive to completion of test.

TABLE 3*

INDIVIDUAL SPUR DEVELOPMENT DURING THIOURACIL TREATMENT AND AFTER ITS INTERRUPTION, SHOWN IN COMPARISON WITH THE NORMAL

Treated					Control			
Age (Days)	Diet (Days)	*D*† (Mm.)	Lengths		Age (Days)	*D*† (Mm.)	Lengths	
			Core (Mm.)	Spur (Mm.)			Core (Mm.)	Spur (Mm.)
211	0	4	14	18	211	4	8	12
358	125	5	18	21	358	0	24	27
578	345	4	20	45	578	0	33	46
695	452	5	21	50	695	0	32	51
758	515	6	22	56	758	0	35	53
	None‡							
817	56	6	23	60	817	0	36	60
887	126	7	27	62	887	0	42	61
943	182	5	29	66	943	0	42	61

* Both birds were 7½ months of age at the start of test and were selected for initial identity of *D*.

† *D* is the distance separating the core of the spur from the tarso-metatarsus.

‡ Days on standard diet below.

and spur core growth in the control are compared with those in the test fowl both during the time of thiouracil administration and for a period after the drug was discontinued.

EFFECTS OF DIETARY SUPPLEMENTS FED IN CONJUNCTION WITH THIOURACIL

The selection of the five supplements used was based on their presumed ability to counteract the established effects of thiouracil in the spur. Each was added to the dry mash containing 0.5 per cent thiouracil as follows: calcium, 2.5 per cent (calcium carbonate); phosphorus, 2 per cent (disodium phosphate); calcium and phosphorus, 5 per cent (dicalcium phosphate); vitamin D, 0.5 per cent (D-activated animal sterol, "Delsterol," 4500 iu/lb); manganese sulphate, 0.044 per cent. Four young but adult cocks were assigned to each substance, and the birds were so distributed that similar initial stages of spur development were represented in each group. The tests were made concurrently. In each set, as survival permitted, birds were kept upon the combined diet for 10 or 15 months; the thiouracil was then omitted but the previously added supplement continued. Survival of the birds, counted in months from the inception of the test, was as follows: calcium, 12, 22, 22, 25; phosphorus, 11, 16, 18, 26 (bird killed); calcium and phosphorus, 10, 25, 26, 26 (birds killed); vitamin D, 7, 10, 14, 26 (bird killed); manganese sulphate, 11, 16, 18, 26 (bird killed).

The findings in this experimental series may be summarized by saying that none of these five supplements altered the course of development in the spur to any extent; these proceeded more or less as if in the presence of thiouracil alone. However, the addition of vitamin D appeared actually to intensify the action of the drug. On the other hand, calcium and phosphorus, continued after the administration of thiouracil was interrupted, seemed to favor a distinctly improved recovery.

EFFECTS OF HORMONES GIVEN IN CONJUNCTION WITH THIOURACIL

Thyroxine, theelin, and testosterone were given separately by injection to simultaneously thiouracil-fed fowl. Thyroxine was chosen for its anticipated correction of the thiouracil-induced effects; the androgen and the estrogen because of the part they are each supposed to have in the spur development of normal fowl. The hen, as a general rule, lacks spurs, although spurred individuals are observed from time to time with, apparently, no diminution of female reproductive activity. The situation in the hen is curious, for it suggests that, once spur proliferation is set in motion for any reason, it is no longer subject to restraint by the ovarian secretions. This implies the presence, in these processes also, of critical stages similar to those which have been found of such importance in the thiouracil-induced spur modifications. The combined administration of the estrogen and thiouracil is therefore of interest.

Spur length in the adult cock is stated to be exceeded by that of the capon (Goodale, 1917), and growth and maturation proceed more rapidly in the young castrate (Quigley and Juhn, 1951). If the slower development of the spur in the cock is due to a slight but positive retardation by the testes, the effects of testosterone and thiouracil might prove additive in the reaction elicited.

The three sets of experiments were started simultaneously in birds of similar ages. Four cocks were assigned to each test, the individual members so chosen that comparable initial spur stages appeared in every group. The selection was

made from a study of X-ray photographs of the spurs of a larger group of untreated birds. These photographs were taken just prior to the commencement of treatment, and similar records were obtained at intervals during the entire period of observation. Thyroxine (crystalline throxine, Squibb) was given twice weekly by subcutaneous injection in doses of 1.0 mg. in 0.5 ml. distilled water. Theelin and testosterone were each injected subcutaneously daily as 0.5 mg. dissolved in 0.20 ml. corn oil, or the equivalent amounts were distributed thrice weekly. The combined treatments were given for 1 year and 1 month, the thiouracil was then omitted, and the concurrently administered hormone was continued alone for 4 months. Thereafter, survivors were observed untreated until disposed of by autopsy, 4 months later.

The choice of dosage in which the three hormones were used was based arbitrarily on earlier experience with these substances in nonthiouracil-treated fowl of comparable age and sex. Given over protracted periods, 1.0 mg. thyroxine twice weekly approaches the limit of tolerance. One-half mg. theelin per day is adequate for general feminization of the male plumage; testosterone was arbitrarily given as weight equivalent of the estrogen.

The selection of these concentrations proved fortunate, in that fairly clear-cut findings were made in the spurs. The addition of thyroxine, quite irrespective of initial spur stage, favored an essentially normal development, as was anticipated (see Pl. IV, Fig. 23, and compare with Pl. II, Fig. 10). Both theelin and testosterone very slightly intensified the thiouracil effect, in that spur stages somewhat more advanced in development responded than had been found the case with the drug alone. The large amorphous structures appearing at the normal locus of the socket, which are typical of early and prolonged thiouracil treatment, also developed in the additional presence of testosterone. The very gradual process is particularly well seen in the individual selected for illustration in Plate IV. The series of X-ray films reproduced gives a view of the entire sequence of events. Figure 22 shows the normal initial spur stage, Figure 25 the appearance after approximately 1 year of the combined thiouracil-testosterone treatment. Figures 26 and 27 show the spur developments at 2- and 4-month intervals, respectively, of testosterone given alone. Figure 28 illustrates the final state, 4 months after the testosterone injections had also been interrupted. An examination of the photographs will show that continuation of the androgen alone did not uphold the earlier effects obviously deriving primarily from the concurrently administered thiouracil. For during the period of testosterone injection alone, connections slowly were made between the surface of the tarsometatarsus and the shapeless mass representing the socket (Pl. IV, Figs. 26, 27). The dorsal junction became completed first, as appears characteristic of the processes of union and of repair in general. A ventral cleft was still present at the final inspection (Pl. IV, Fig. 28), just prior to autopsy, and almost 2 years from the time of the first observation.

EFFECTS OF ESTROGENS ON SPUR STRUCTURE

A special investigation was made of estrogens alone, because of the normal inhibition of spur growth by the ovary, which was discussed earlier. Twenty young New Hampshire cocks were assigned to this test. Seven received 3.5 mg. theelin in 1.40 ml. corn oil distributed in three weekly subcutaneous injections; six received stilbestrol in identical dosage; and seven were continued untreated.

X-ray photographs of the spur were taken prior to the distribution of the fowl to the three groups and were obtained again at bimonthly intervals thereafter. Particular attention was given the plumage for evidence of female modification. Treatment was continued for about 13 months, with good survival. During the last month the dosage of stilbestrol was raised to 20 mg. per day in two birds and to 20 mg. theelin in one. Survival was 21 and 22 days in the stilbestrol-treated cocks, and 27 days in the single theelin-injected cock. Incoming feathers always showed the expected female alterations, but an examination of the series of X-ray films proved both the natural and the synthetic estrogen to be entirely negative in the spur. Structural development and growth proceeded as in the normal at all dosages.

EFFECTS OF RELAXIN IN THE SPUR

Relaxin was tried in a small number of birds because the peculiarly late processes of union between the spur core and the shank bone could offer sufficient local flexibility for a response here. The relaxin used was a preparation containing 200 guinea-pig (g.p.) units per milliliter and with no estrogenic properties. It was tested for its effect on the spur of adult, estrogen-injected cocks as follows:

A. Two cocks, pretreated and continued upon 0.5 mg. theelin per day, received 500 g.p. units at 8:00 A.M. of one day and again at 4:30 P.M. of the following day. Injection of the relaxin preparation was made subcutaneously in the leg above the spur, and the fluid was gently massaged downward. X-ray photographs were taken the day of the first injection and every other day thereafter for four exposures.

B. Two cocks on the same estrogen treatment were similarly injected, but with 20 g.p. units each morning and afternoon for 28 days. X-ray photographs were obtained weekly.

The films of each series showed that relaxin did not cause a separation of the spur core and the tarso-metatarsal surface under the conditions of the experiment.

COMPARISON OF SPUR DEVELOPMENT IN THE PRESENCE OF THIOURACIL IN THE COCK, SLIP, AND CAPON

Domm and Blivaiss (1948) noted the manually perceptible loosening of the spur among some of their cocks fed thiouracil for 9–10 months but failed to find this in capons similarly treated. These observations differ from records from this laboratory (Juhn, 1946, 1948), and the differences cannot well be referred to the respective dosage levels of thiouracil employed. Calculated from feed-consumption records, the daily dosage of thiouracil received by adult experimental fowl in this laboratory is *ca.* 0.4 gm. (Juhn, 1944*b*). Domm and Blivaiss' birds ingested 0.5 gm. daily, which is about 25 per cent more.

The earlier studies from this laboratory did not make full use of the X-ray photography upon which primary reliance is now placed in judgment of spur condition. Therefore, it was decided to set up comparisons of the effects of thiouracil in the spur of the cock and capon and to extend the observations to include the slip. The birds used were all of one day's hatch of closely related New Hampshire chicks, and they were raised under identical conditions. Caponization or partial castration (slips) was performed at 6 weeks of age. The birds were transferred from the range to individual cages at 1 year of age. X-ray photographs of the spur region of the entire lot of eight cocks, five slips, and seven capons were taken at that time. The birds were then placed on the usual thiouracil diet,

and all the later records were also kept as in the previous experiments. The observations were continued for approximately 10 months.

Where treatment was commenced at suitably early stages, spur-core separation from the tarso-metatarsus continued in individuals of all three classes of thiouracil-treated fowl. However, successive steps in structural differentiation are attained more rapidly in spurs of the capon than in the cock, with the slip occupying a somewhat intermediate position (cf. p. 156). Hence an entirely different composition with respect to this physiological response may obtain among operated and normal fowl of identical age. The importance of an initial internal inspection is therefore indicated.

EFFECT OF THIOURACIL IN THE SPURRED HEN

Two females were available for observation. In the first of these—a White Leghorn—short (22 mm.), very stout spurs were present. X-ray photographs showed this spur to be composed of a very large and heavy core with narrow epidermal covering. This core was in union with the shank, without perceptible development of a socket; the entire proximal segment was slightly narrower in proportion and less heavily calcified. The hen was fed thiouracil for 13 months, and the spur condition was recorded as in preceding experiments. The bird continued in good shape at all times. During the period of treatment the spur showed slight external changes, it became upward-curved and even thicker, but the length increased only little. The core expanded in conformation and remained attached to the tarso-metatarsus. A truly enlarged socket was not formed.

The second hen—a New Hampshire—had long (43 mm.), delicate, and narrow spurs. The core, 30 mm. in length, was separated from the shank by a distance of 4 mm. Proliferation of the socket had apparently just commenced. This bird was fed thiouracil until its death, 9 months later. During this time the length of the spur increased to 54 mm. Spur-core length diminished slightly and increased again to the initial figure. The socket primordium developed somewhat further, but the distance between it and the core was not bridged.

Of the two female spurs studied, one chanced fortunately to be very much less advanced in development than the other. The comparison of their individual later growth in the presence of thiouracil shows that the spur of the hen responds as does the spur of the cock when compared stage for stage.

DISCUSSION

The chief interest of these investigations on the effects of thiouracil in the spur of the adult male fowl resides in the peculiar developmental aspects which these experiments have illuminated. The diminution in titre of circulating thyroid hormone caused by the continuous presence of the drug (Astwood, Sullivan, Bissell, and Tyslowitz, 1943) is reflected far less in growth than in differentiation. Although growth of the male spur is apparently continuous from early periods onward, differentiation of the central core and the following connections with the tarso-metatarsus are initiated only in the adult. The appearance of these developments at a time when the bird has come to full maturity is remarkable. Even more remarkable is the capacity for induction, at this stage of life, which characterizes the spur core. Origination of the socket from the shank by induction was clearly envisaged by Kozelka (1933, p. 73), in referring to his own unpublished observations. He appears to have attributed this property to the entire spur, which would give equal importance to

the ectodermal components. The present studies, however, indicate that the capacity for induction is an attribute of the spur core, a derivative of the mesenchyme.

Induction is nullified when thiouracil is present from suitably early stages onward, and the role of the drug in this respect is of prime importance in later spur development. When formation of the socket is not initiated, the supporting architecture of the spur is lacking. The existing membranous attachments to the shank then become insufficient to sustain the weight of the lengthening spur. The oversize, shapeless masses that develop at the socket locus when growth is resumed after an earlier interruption by thiouracil further indicate the importance of the thyroid hormone for differentiation at critical stages. Vitamin D, testosterone, and theelin all appear to underscore slightly the effects of thiouracil, but there is little evidence that any one of these three substances would, of itself, duplicate the action of the drug. The effects of thiouracil are indirect, in the sense that all appear to derive more properly from the diminution in output of the bird's own thyroid; this is in harmony with the essentially negative findings made with the supplemental substances used besides thyroxine. It is, of course, possible that the depression of the thyroid may favor an accelerated or new release of some substance which actively inhibits induction and differentiation in the spur, but evidence for this did not develop in the present experiments.

The situation in the spur of thiouracil-treated fowl with its continued but disorganized growth is curiously reminiscent of observations made on entirely separate material. Wislocki, Aub, and Waldo (1947) give an interesting account of the consequences of castration and of the implantation of testosterone propionate pellets at different ages and seasons on the antlers of the Virginia deer. In the normal course of events, the antlers are cast off in midwinter, and growth recommences in the spring. Shedding of the antlers occurs at a period of declining testicular activity, and renewal coincides with its nadir.

When a fawn is castrated prior to any antler origins, there are no later developments. If the operation is performed in the mature individual, the annual cycle of loss and renewal becomes obliterated. The antlers are retained indefinitely, and a continuous but disorderly growth leads to bizarre formations. Such animals may be caused to shed these protuberances by the implantation of testosterone pellets, but the replacements continue to be of the same castrate character. Antler growth can be initiated in deer castrated prior to their appearance by similar implants. This growth, once stimulated, will continue in the absence of further androgen administration, but it will also be of a disorganized nature.

The role of the thyroid in spur development appears closely comparable to that of the androgen in antler formation. Growth as such proceeds in the lack of either hormone, but structural differentiation is not attained. There is nothing equivalent in the spur[4] to the shedding and renewal of the deer's head furnishings, but annual or semiannual fluctuations are recorded in the normal molts of the plumage. The timing of these events appears also to bear little relation to secreted androgens but to depend more di-

[4] Mr. Elliot Kimball, Clinton Experimental Farm, Clinton, Connecticut (personal communication), has, however, under observation a Brown Leghorn cock which, on or about the time of the annual molt, also sheds his spurs. The bird is otherwise normal, now something over 6 years of age, and new, cone-shaped spurs were regenerated on five separate occasions to date.

rectly on levels of thyroid activity. Certain functional aspects of the one gland in a lower form would seem to be taken up by another with increasing complication, perhaps as a part of the evolutionary process.

SUMMARY

The effects of thiouracil on internal spur differentiation of the adult cock were investigated with particular reference to the stage of development of the spur, as well as to the age of the fowl at the inception of treatment.

As a basis, the normal processes of spur calcification were first established. Organization of the central bony core was found to take place within the already well-grown spur of the cock. The core originates at a distal point in the spur, and growth proceeds toward the tarsometatarsus. The expanding core, at a certain critical point in its advance, induces the proliferation of the socket from the shank. The special course of these developments is of first importance in the modifications caused by thiouracil in the spur. Noncompletion of core-shank junction, absence of socket formation, and retardation of core growth all depend on the spur stage at inception of treatment.

Concurrent administration with thiouracil of calcium, phosphorus, calcium and phosphorus, manganese sulphate, and vitamin D showed little difference from thiouracil given alone.

Thyroxine, theelin, and testosterone were given by injection to simultaneously thiouracil-fed cocks. Thyroxine offset the action of the drug; the sex hormones slightly intensified it. Theelin, stilbestrol, and relaxin given alone did not duplicate the action of thiouracil.

It is concluded that the effects of thiouracil in the spur are primarily due to the developmental retardations it causes and that these various retardations are possible in the spur because of the uniquely late phases of its maturation.

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PLATE I

An example of the cystic enlargement of the thyroids in a thiouracil-fed cock is shown in comparison with the normal glands from a cock of similar age and similar other conditions of management. The treated bird had received 0.5 per cent thiouracil in the diet for about 1 year prior to autopsy. Photographs by Leet Brothers & Co., Washington, D.C.

FIG. 1.—Left gland, treated bird, weight 158.7 gm.

FIG. 2.—Left gland, normal bird, weight 0.24 gm.

FIG. 3.—Right gland, treated bird, weight 144.6 gm.

FIG. 4.—Right gland, normal bird, weight 0.25 gm.

PLATE II

Normal stages of spur calcification in the cock as recorded in X-ray photographs.

FIG. 5.—♂, 9 months. No calcification. Spur length, 13 mm.

FIG. 6.—♂, 7 months. Discrete granule, 4 mm. distant from the tarso-metatarsal surface. Spur length, 12 mm.

FIG. 7.—♂, 7 months. Organization of central core primordium. Spur length, 12 mm.

FIG. 8.—♂, 9 months. Distinct central core present, 10 mm. in length, 4 mm. distant from the tarso-metatarsus. Spur length, 17 mm.

FIG. 9.—♂, 9 months. Core increasing in length (25 mm.) and approaching the tarso-metatarsus. Spur length, 32 mm. Note beginning of bony proliferation from the shank toward the spur core.

FIG. 10.—♂, 18 months. Mature spur with socket enveloping the core base. Core length, 26 mm.; spur length, 37 mm. Calcification of the proximal socket segment will become more dense in later life of fowl.

PLATE III

Spur development in the presence of thiouracil and after interruption of treatment in comparison with the normal. The paired cocks were from one day's hatch of the same mating and were selected for initial identity in the distance separating the spur core from the tarso-metatarsus. All figures are X-ray photographs.

NORMAL ♂

FIG. 11.—Age 211 days.

FIG. 12.—Age 358 days.

FIG. 13.—Age 578 days.

TREATED ♂

FIG. 14.—Age 211 days; days thiouracil, 0.

FIG. 15.—Age 358 days; days thiouracil, 125.

FIG. 16.—Age 578 days; days thiouracil, 345.

FIG. 17.—Age 686 days; days thiouracil, 453.

FIG. 18.—Age 817 days; days after cessation of treatment, 56.

FIG. 19.—Age 943 days; days after cessation of treatment, 182.

PLATE IV

Spur formation in thiouracil-fed cocks simultaneously treated with thyroxine, with theelin, and with testosterone. All figures are X-ray photographs.

THIOURACIL + THYROXINE, ♂ 761

FIG. 20.—Normal initial stage.

FIG. 23.—Approximately 1 year later.

THIOURACIL + THEELIN, ♂ 721

FIG. 21.—Normal initial stage.

FIG. 24.—Approximately 1 year later.

THIOURACIL + TESTOSTERONE, ♂ 726

FIG. 22.—Normal initial stage.

FIG. 25.—Approximately 1 year later.

THIOURACIL OMITTED, TESTOSTERONE CONTINUED

FIGS. 26 and 27.—Two and 4 months later.

FIG. 28.—Four months after all treatment was interrupted.

PLATE I

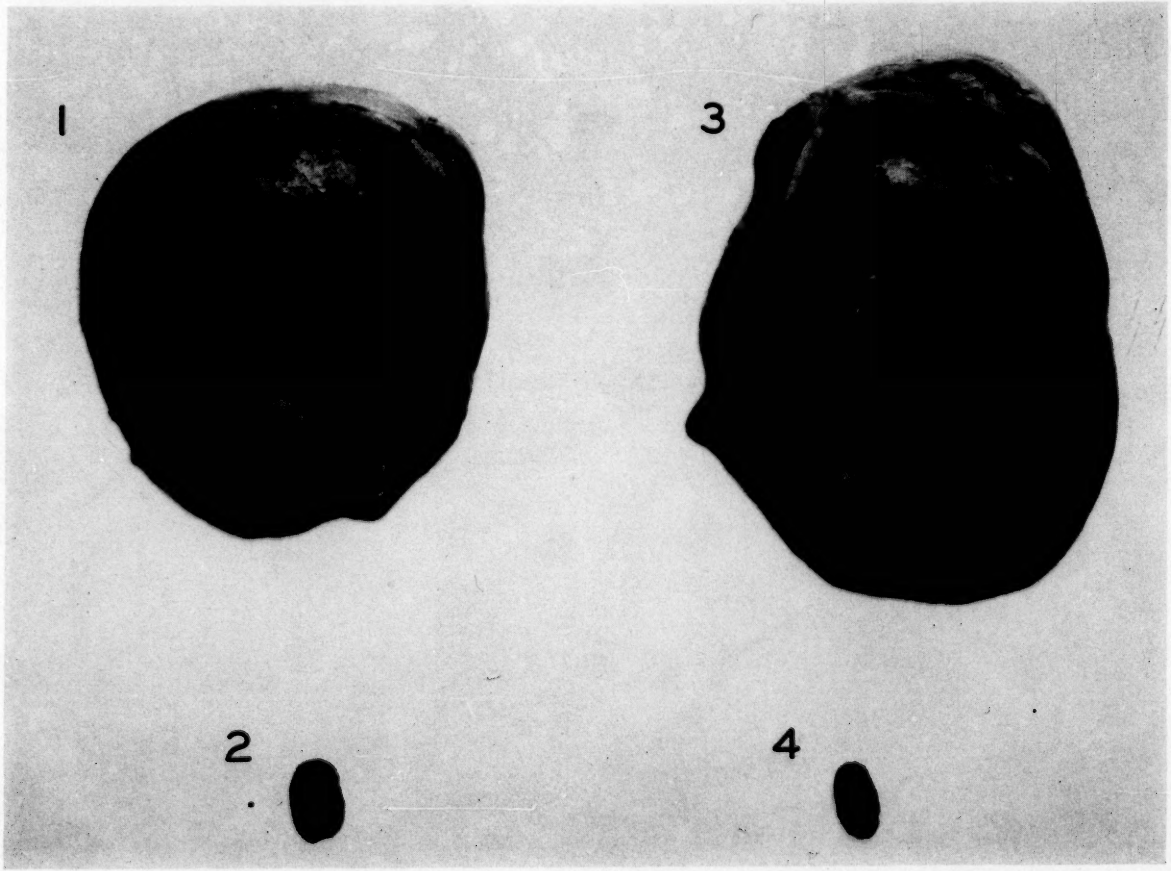


PLATE II

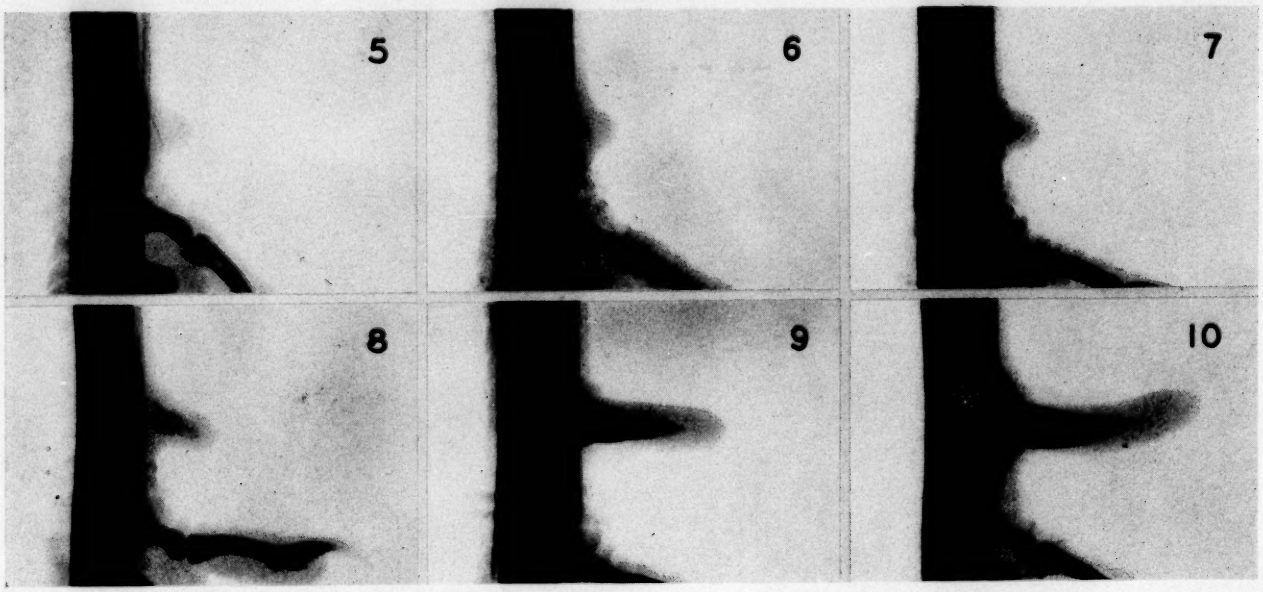


PLATE III

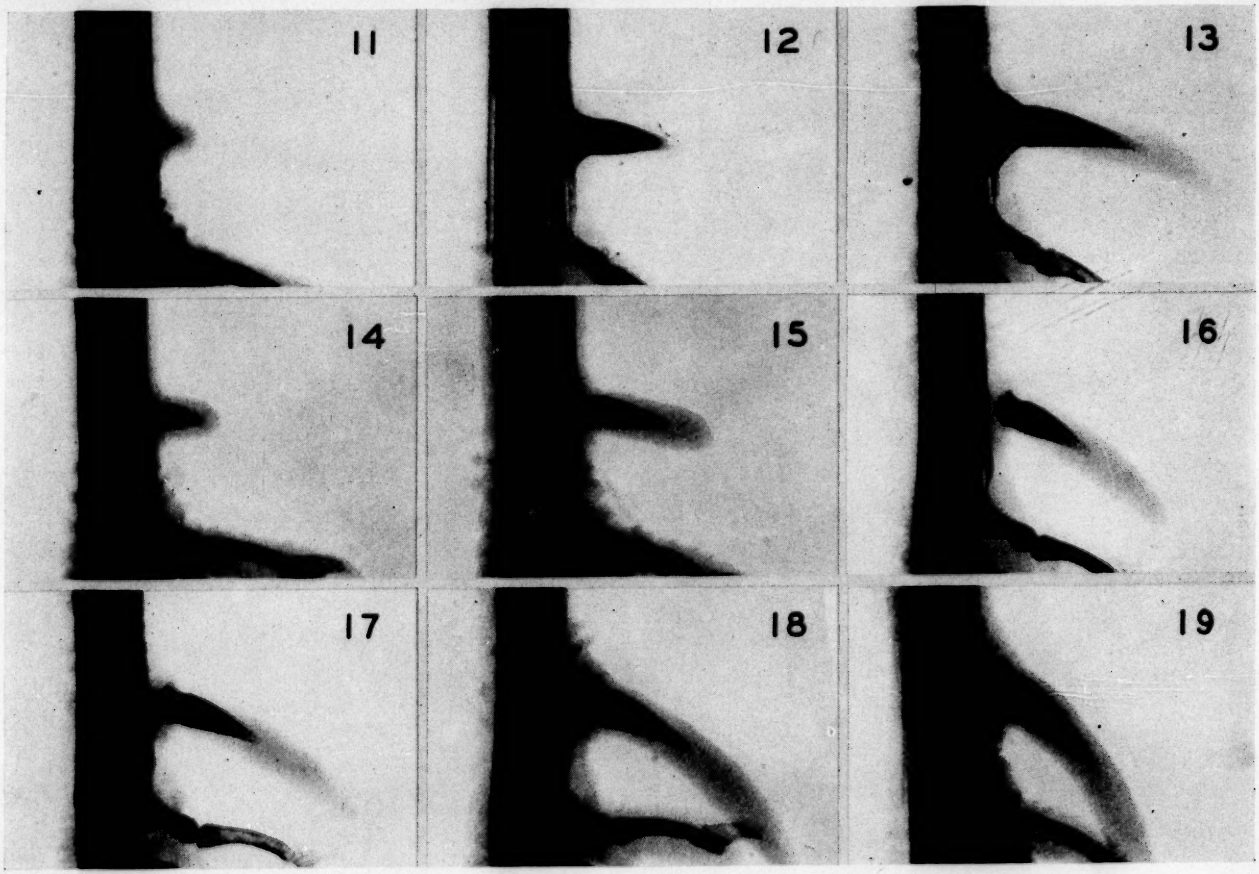
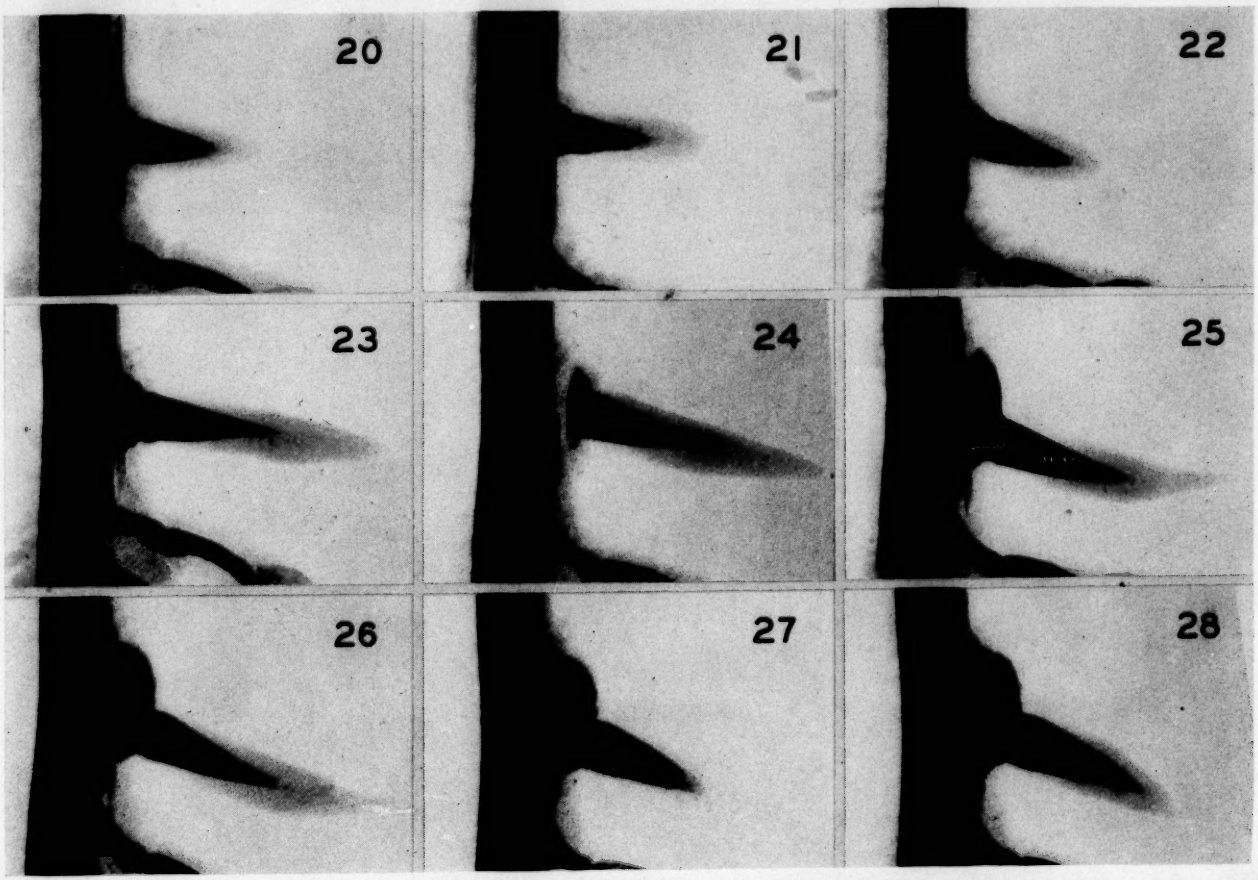


PLATE IV



LOCAL METAMORPHOSIS OF LARVAL SKIN IN RANA PIPIENS

JERRY J. KOLLROS[1] AND JANE C. KALTENBACH[2]
Department of Zoölogy, State University of Iowa, Iowa City, Iowa

MANY hormones normally reach the site of their action via the blood stream, but under certain circumstances it is desirable to apply them locally, in the form of pellets, capsules, ointments, or solutions. The major reasons for local application are (1) to obtain slow absorption of the hormone from its carrier agent; (2) to obtain local concentrations of the hormone that are higher than those safely or easily obtained by other means; (3) to obtain specific effects locally without exposing the remainder of the body to the hormone (see Speert, 1948, for a review of local actions of sex hormones).

The techniques of local application can be employed profitably in amphibian larvae for the analysis of the metamorphosing action of thyroxin or of its analogues upon specific tissues at specific sites. Hartwig (1940) obtained reduction of the dorsal fin and histological changes in the dorsal skin of *Salamandra* larvae in the vicinity of thyroxin implants. His hormone source was an agar pellet which had been soaked in a solution of thyroxin. Similar agar-thyroxin pellets, as well as fragments of fresh rat thyroid gland, were employed by Kollros (1942, 1943) in demonstrating the dependence of the corneal reflex center of the anuran tadpole for its maturation upon thyroid hormone. Lüke (1944) also showed local metamorphosis of salamander skin in the vicinity of subcutaneous implants of axolotl thyroid gland. Localized metamorphic changes involving the eyeball and its adnexa, mouth furnishings, skin, skin window, hind limbs, and head symmetry in *Rana pipiens* have been reported by Kaltenbach (1949, 1950, and in preparation). Changes in size of certain brain cells, parallel to those occurring in normal metamorphosis, have been induced by thyroxin-containing pellets placed in the intracranial cavity of frog tadpoles; the changes involved cells of the mesencephalic V nucleus (Kollros *et al.*, 1950), and Mauthner's cell (Weiss and Rossetti, 1951).

Since most anuran tissues require several days of exposure to moderate concentrations of thyroxin before metamorphic changes can be detected, it is desirable for the thyroxin-containing implant to yield its hormone continuously for a period of several days or even weeks. Experience with agar soaked in thyroxin solutions and with fresh mammalian thyroid gland has revealed that these sources yield hormone to the surroundings very rapidly (Adams and Merwin, 1939, report complete loss of follicular colloid from mouse thyroid glands within 24 hours of their transplantation into *Triturus*). The hormone enters the body fluids and is quickly distributed to all parts of the body, so that local changes tend to be masked by the rapid metamorphic changes occurring in the entire body. In order to retard loss of thyroxin from the implant, pellets were manufactured from mixtures of thyroxin crystals and cholesterol. Experience with

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[2] Present address: Department of Pathology, Medical School, University of Wisconsin, Madison, Wisconsin.

such pellets has demonstrated their superiority to those previously used. The hormone dose can be measured accurately, and the thyroxin is yielded to the surroundings for periods of 7–10 days or longer. This rate is sufficient to permit fairly rapid acceleration of metamorphic changes in the immediate vicinity of small pellets, yet slow enough so that only a moderate acceleration of metamorphosis of the whole animal can be detected. Thus asynchronies develop between the metamorphic rates of locally treated and of untreated regions of the body. The present paper illustrates the use of the thyroxin-cholesterol pellets in demonstrating the direct dependence of skin changes upon thyroid hormone.

MATERIAL AND METHODS

The *R. pipiens* tadpoles used in these experiments were raised from eggs collected in ponds in the vicinity of Iowa City, Iowa, and from eggs obtained by artificial fertilization according to the method of Rugh (1934). The tadpoles were raised in groups in large containers. They were anesthetized in a 2 per cent solution of ethyl urethane and were operated upon in Holtfreter's solution, with the aid of a binocular dissecting microscope. After operation, the tadpoles were placed in individual glass containers. Both operated and stock animals had boiled lettuce available at all times. The developmental level of the tadpoles was determined according to the staging plan of Taylor and Kollros (1946).

Subcutaneous implantation of pellets was accomplished by making a short slit in the dorsal skin and producing a tunnel under the skin by the use of a probe. The pellet was placed in the slit and pushed to the end of the tunnel. In this way the pellet came to lie under essentially undamaged and nonregenerating skin, a few millimeters away from the slit. A control pellet was inserted in a similar fashion under the skin of the opposite side of the back. A second site for implantation was selected in conjunction with studies involving local metamorphic changes in the midbrain (Kollros *et al.*, 1950). The pellet was placed in the cranial cavity just anterior to the right optic lobe, approximately 1–2 mm. below the skin surface and directly below the small wound area which was made in exposing the brain. In this second site, regenerating skin as well as surrounding "normal" skin came under the influence of the hormone from the pellet.

The pellets were manufactured from mixtures of thyroxin crystals and cholesterol, in a ratio of 1 to 4 by weight. The mixture was compacted into pellets of 0.5-mm. diameter by a modified Hartman apparatus (Hartman, 1940; Kaltenbach, in preparation). Standardization of pellet composition was obtained by thorough mixing of the components and by the use of a uniform number of blows of the "hammer" during the process of compaction. The pellets weighed about 0.3 mg. per millimeter of length. Control pellets of cholesterol were produced in a like manner. Fragments of from 0.15 to 0.35 mm. in length were used as implants.

Tadpoles were fixed in Bouin's fluid, dehydrated in alcohol, imbedded in paraffin, and sectioned at a thickness of 10 μ. The sections were stained with Ehrlich's acid haematoxylin and eosin. Slides of previously prepared tadpoles of a "normal" series were also available for comparisons.

NORMAL DEVELOPMENT OF SKIN GLANDS

The development of skin glands, particularly in the dermal plicae, has been studied in *R. sylvatica* by Helff and Stark (1941). Approximate translations of their stages to those used for *R. pipiens* indi-

cate that gland development is a late larval feature. In *R. sylvatica* solid gland nests are usually developed by stage XV, and small patent glands by stage XVII. By stage XVIII the stratum spongiosum, previously absent, appears in the region of the largest glands. At stage XX the epidermis is clearly three-layered, in contrast to its two-layered larval condition. In the control slides of *R. pipiens*, gland nests are absent in the single cases studied of stages XI, XII, and XIII. In stage XIV only very small gland nests are present. By stage XVII, small patent glands and a thin stratum spongiosum have developed. In stages XIX and XX the stratum spongiosum is a continuous, thin layer; the epidermis in these stages has three layers.

RESULTS

A. SUBCUTANEOUS IMPLANTATION

Eleven tadpoles in stages II, III, and IV (average in III) had thyroxin-cholesterol pellets implanted subcutaneously in a position medial to and just posterior to one eye. Cholesterol control pellets were implanted subcutaneously in a symmetrical position on the opposite side. In three animals the pellets were expelled through the skin within 3 days. These three received new thyroxin-cholesterol pellets 21 days after the initial operations. Since the skin changes following the second implantation were of the same nature as those of the original group, they will not be described separately.

The adult frog has many round or oblong dark spots on its dorsal surface. Each of these is bounded by a rim of much darker pigment, and this, in turn, is usually surrounded by a "halo" of pigment which is slightly paler than the background color of the surrounding skin. At 8 days after operation, when the animals had advanced to stages V–VIII, the pigment spot, which usually develops medial to the dermal plica and just posterior to the eyelid, was first discernible as a ring of pigment slightly darker than the adjacent skin. Just posterior to this spot a grayish or faded streak could be seen, marking the premature development of the dorsolateral dermal plica. These pigmentation changes were unilateral and were limited to the area immediately above and surrounding the thyroxin-cholesterol pellet.

By 14 days after the operation, rapid metamorphic progress had ended. The tadpoles were in stages VII–X. Within the next 2 weeks they developed slowly to stages VIII$^+$ to XIII. With the exception of one tadpole which entered metamorphic climax less than 3 weeks after receiving its second implantation, all the tadpoles entered a period of metamorphic stasis. Some recovered from stasis rapidly and some slowly or not at all.

The four animals which emerged from stasis in 1–3 weeks were fixed during metamorphic climax at 65–127 days. During stasis and afterward they showed advanced skin changes in the vicinity of the pellet. The skin spot darkened considerably, and, by 40 days after the operation, molting of the skin in the restricted area above the pellet could be seen. In three cases the dermal plica was restricted to a short, pale, elevated area, just posterior to the pellet; but in one instance the plical ridge extended caudad a distance of about 3 mm. and was elevated more than in the other cases, giving evidence of the more advanced level of development of its glands. The skin pigmentation and the dermal plica were maintained at the most advanced level they had achieved until the opposite side of the animal showed equivalent metamorphic change; after that, both sides appeared to metamorphose together. By stage XXI or XXII, when the animals

were fixed, almost all indications of bilateral disharmony had disappeared.

The other six larvae, including the two most highly stimulated ones, were sacrificed in stages X–XIII, at various intervals from 32 to 227 days after operation. At the time of sacrifice they were either still in the stasis stage or just emerging from it. The pigment spots were clearly defined in the region of the hormone pellet. By 21 days the spots were encircled by pale areas, and by 27 days some guanophores had developed within the pale rings. Local molting could also be seen at this time. The pale streak of the dermal plica extended slightly caudad of the pellet in the early stasis period, and, as glandular development proceeded within it, a low plical ridge appeared. In four animals all these changes were unilateral, on the side of the hormone pellet.

Cross-sections through the implant area reveal either moderate or marked metamorphic stimulation of the skin just above the pellet. A gradient of decreasing metamorphic effect with increasing distance from the pellet is obvious. Molting is limited to skin in the close vicinity of the hormone source. In the area of molting and for a very short distance beyond it, patent skin glands and stratified epidermis can be seen. More peripherally, gland nests of progressively smaller size appear. Some 2–3 mm. away from the pellet the gland nests no longer are found, and the spongy stratum of the dermis is also absent. The dermis then shows its typical larval condition of a compact stratum only. In the region of the smallest gland nests, the Leydig cells of the epidermis can be seen. These typical larval cells are completely absent from the area near the hormone pellet, and, in fact, their disappearance is one of the earliest signs of local hormone effect. Restriction of the stimulated area in this group of stasis tadpoles indicates that the hormone level in the circulating body fluids was generally inadequate to stimulate the skin over the whole body. One can also assume that the circulating blood and lymph in the vicinity of the implant did take up the hormone and, by carrying it away, restricted its effective local diffusion to a zone of a few square millimeters.

In the two most highly stimulated animals, changes were bilateral, presumably as a result of hormone in the circulation affecting skin structures on the "control" side of the body. But the changes on the "control" side were far less marked than were those on the side containing the hormone pellet. The plical ridges, for example, were well raised on the highly stimulated side, and barely perceptible on the opposite side (Pl. I, Fig. 1). Even in these animals, evidence of a gradient of thyroxin influence was clear.

B. INTRACRANIAL IMPLANTATION

Twenty-three tadpoles, eleven of which had been hypophysectomized at the tail-bud stage, received intracranial implants of thyroxin-cholesterol pellets just anterior to the right optic lobe. These pellets were effective in bringing about metamorphic changes in the scattered cells of the mesencephalic V nucleus. The degree of stimulation was inversely proportional to the distances from the pellet to the cells (Kollros *et al.*, 1950). The pellets were also effective in bringing about metamorphic changes in the skin of the head. Since the degree and extent of the local changes depended more upon the length of the period of stimulation than upon the amount of thyroxin implanted, the size of the implant will not be mentioned in individual cases. In addition to the local changes, sufficient hormone entered the blood stream to be effective in inducing a generalized advance in development. Tad-

poles were initially in stages III through VI and were fixed at intervals of 2 to 20 days in stages III through XIII.

Two hypophysectomized animals were fixed 2 days after operation, in stages III and III+. A barely perceptible increase in leg size was detected in the larger of the animals. There were no metamorphic changes in the skin. The wound had healed, and the epidermal cells which had accumulated were just becoming arranged into two layers. The outer cells were flattened, as is typical of the normal skin adjacent to the wound, but the inner cell layer was still disarranged. Cells of this inner layer, however, did not appear to be typical Leydig cells; they stained more darkly and were nearly cuboidal in shape (Pl. I, Fig. 2).

Two hypophysectomized animals were fixed 3 days after operation. They had advanced from stages V+ and VI− to stage VII−. In addition to this evidence of generalized hormonal stimulation, they displayed local skin changes. In one animal cross-sections showed gland nests of minimal size, approximately three to six cells each, in the area immediately adjacent to the wound. The other animal displayed somewhat larger gland nests, both in the circumference of the wound, and in the adjacent skin covering the right orbit (Pl. II, Fig. 3). The larval Leydig cells had also disappeared from the skin closely surrounding the wound but were present within 0.5 mm. of the wound edge. There was no trace of a stratum spongiosum.

Four hypophysectomized animals were fixed either 5 or 6 days after operation. In this period they had advanced from stage III to stages VI through VIII−. Gland nests were slightly better developed than at 3 days. In one instance they developed unilaterally only. In the other three animals moderately well-developed nests were present in the wound area and above the right orbit, while only gland nests of minimal size were present above the left orbit. Two other hypophysectomized animals operated upon at stage VI− were fixed 5 days later at stage IX+. In one case gland nests were restricted to the operated side; in the other case nests of minimal size were present just medial to the left orbit, while well-developed nests, almost large enough to display a lumen, were present in the wound area and in the skin above the right orbit, adjacent to the wound site. In both these animals a thin stratum spongiosum had developed in the restricted area of the largest gland nests.

A single hypophysectomized animal, fixed 8 days after operation, advanced from stage VI− to XII−. Except for the presence of a few glands with small lumina, the skin of this animal was much like that of the animals in the immediately preceding group. In contrast, two "normal" animals, fixed 9 days after operation, following a change from stage VI− to stage X, showed larger numbers of patent glands, a very gradual reduction in size of glands and nests with increasing distance from the wound, and an ipsilateral restriction of these changes. In one animal cornification of the outer epidermis in the region immediately overlying the pellet could be seen, but no molting had occurred (Pl. II, Fig. 4). The stratum spongiosum was still present only in the small area containing the largest glands and gland nests.

Six animals, initially in stage VI, were fixed 10–12 days after the operation, when they had developed to stage XII. These tadpoles showed many relatively large mucous glands opening to the surface in the skin of the wound area, and especially in the supraorbital skin of the right side. On the left side, only a few patent glands could be seen near the midline, while lateral to this were gland

nests, showing a mediolateral size gradient. At the level of the pellet, Leydig cells were absent from the dorsal skin on the right side and present on the extreme left side only, although several millimeters anterior and posterior to the wound region they extended across the mid-line. The stratum spongiosum was thicker than in any tadpoles described previously, but peripherally it remained very thin, being absent far laterad. A thin layer of molted skin was seen in a small region just above the pellet in two animals.

Four other animals, initially in stages IV–VI, were fixed 13–20 days after the operation in stages X–XII. In all these animals the rapid changes in limb appearance which marked general metamorphic progress had come completely or almost completely to a halt, presumably due to the exhaustion of the thyroxin in the pellet and its elimination from the body. Large glands were present above the pellet and in the supraorbital skin on the right side, while the left side showed numerous gland nests, but only a few small glands. The development of the stratum spongiosum was parallel to that of the glands. Leydig cells were completely absent from both sides of the dorsal surface of the head at the level of the midbrain. On the left side the Leydig cells were again present at the level of the midbrain-cerebellar junction, but they reappeared on the right side only at the level of the medulla oblongata. Molting was seen in all animals. In the most extensive case the area of molting extended from the mid-line to the supraorbital region (Pl. II, Fig. 5).

DISCUSSION

The experiments described above show clearly that the metamorphosis of tadpole skin is controlled by the local concentration of thyroid hormone (or thyroxin). In normal circumstances too little hormone is released into the blood by the thyroid gland of the *R. pipiens* tadpole to initiate skin gland development prior to stage XIV. But if pellets containing thyroxin are implanted under the skin even in much earlier stages, gland development can be initiated within a few days. That thyroxin is responsible for this initiation can be seen from the time-space distribution of skin changes and by the absence of *local* changes in the skin above the control pellet. Thus changes in skin morphology, including gland nest formation, occur first in the immediate vicinity of the thyroxin-containing pellet. Later, as the thyroxin influence spreads, the most advanced skin conditions are found immediately adjacent to the pellet, and the less advanced features are found farther away, with intermediate levels being evident in between. The gradation of changes seen in the skin surrounding the pellet shows at one time the sequence of changes which occurred in the skin overlying the pellet.

The decrease in the stimulatory effect with increasing distance from the hormone source indicates a diffusion of the hormone from the pellet. The final limit of stimulatory effect must be determined by the interaction of factors which tend to increase the effective diffusion distance (such as initial amount and concentration of the hormone in the pellet) and to decrease it (particularly the diffusion of thyroxin into blood and lymph capillaries and its consequent rapid transport away from the hormone source). The removal of thyroxin from the pellet area by the circulatory system not only limits the direct local effects but results in generalized metamorphic changes as well. The extent of these broader changes, in turn, is limited by the concentration of hormone in the blood. If the hormone level is low, as with small pellets, only those organs with capacity to react rapidly to low concentrations will show a response,

e.g., the limbs will grow. If the hormone level is relatively high, as with large pellets, there will be a period of lesser or greater duration during which organs with a "high" threshold respond in addition to those with a "low" threshold, e.g., skin glands generally, including dermal plicae on both sides. The events which mark metamorphic climax (spiracular reduction, eyelid and nictitating membrane formation, development of a skin window and its perforation, etc.) were not produced in this study as a result of implantation of a thyroxin-cholesterol pellet. Presumably, the thyroxin concentration in the blood required for the rapid onset of these events was never reached or, if attained, was not maintained for an effective period. That thyroxin levels approaching those required for climax events were obtained is illustrated by those few cases in which skin changes characteristic of the preclimax stages XV–XVI were observed on the side opposite the hormone-containing pellet as well as on the same side.

There is some indication, in series B, that, while normal and hypophysectomized tadpoles show about the same degree of hind-limb development or generalized metamorphic change, there may be some difference in the degree of local response in the skin, with the hypophysectomized animals tending to lag behind the others. The tendency to bilateral but unequal development of the supraorbital glands suggests that the skin of this region is either more sensitive to thyroxin or that it can respond more rapidly to low concentrations of thyroxin than can the skin elsewhere, since the glandular development in the supraorbital skin was commonly superior to that seen in skin nearer the pellet.

It is also fairly clear that, at the levels of dosage employed in this study, minimal stimulatory changes can be noticed within 2 days; that developmental changes show their peak rates in the 3–6-day period; and that changes in the body as a whole are almost at an end 10–12 days after implantation of the pellet, although local changes may continue slowly for some days thereafter.

SUMMARY

1. Pellets of thyroxin in cholesterol were implanted under the dorsal skin or in the cranial cavity of *R. pipiens* tadpoles in order to elicit local metamorphic responses in the surrounding tissues.

2. Skin glands developed prematurely in the immediate vicinity of the hormone pellets. This development was accompanied by other metamorphic changes in the skin near the pellet, including typical changes in pigmentation, development of spots, initiation of dermal plicae, development of stratum spongiosum, and the stratification, cornification, and molting characteristic of the epidermis at metamorphic climax.

3. Maximum metamorphic changes were obtained immediately above and adjacent to the pellet, and these effects decreased with increasing distance from the pellet. Such a pattern of change suggests that this metamorphosis is under the direct control of thyroid hormone.

4. Some degree of generalized metamorphic change was noticed in all cases, indicating the accumulation of thyroxin in the blood and its general distribution thereby. Since no animal developed beyond stage XIII as a direct result of hormone treatment, neither glands nor gland nests would normally be expected. But in a few cases generalized stimulation was sufficient to permit some skin gland development over the whole body, in addition to the marked local development near the hormone pellet.

5. Rapid metamorphic changes, both local and general, were usually at an end within 2 weeks, although local changes might continue slowly for some time thereafter.

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PLATE I

FIG. 1.—Tadpole in stage XIII, 217 days after pellet implantation, ×100. *A*, anterior to thyroxin-cholesterol pellet, showing mucous and granular glands of dermal plica. The otic cartilage is present at ventral border of picture. *B*, area comparable to *A*, but on opposite (control-pellet) side.

FIG. 2.—Tadpole in stage III, 2 days after pellet implantation. *A*, wound area, showing encapsulated pellet adjacent to dorsolateral wall of the diencephalon. Leydig cells appear near the edges of the picture. ×120. *B*, the bracketed area of *A*, enlarged to ×600, showing incompletely organized epidermis and absence of Leydig cells.

PLATE II

FIG. 3.—Tadpole in stage VII⁻, 3 days after implantation. Gland nests are present above the pellet and in the supraorbital skin. The edge of the encapsulated pellet appears at the lower left, and the edge of the eye at the lower right. ×208.

FIG. 4.—Tadpole in stage X, 12 days after implantation. *A*, the encapsulated thyroxin-cholesterol pellet is dorsolateral to the right side of the midbrain. Patent glands appear in the skin above the pellet, and gland nests appear to either side. Note the absence of glands on the left side. ×36. *B*, bracketed section of *A*, enlarged to ×524. Note the well-developed stratum spongiosum and the stratified, three-layered epidermis.

FIG. 5.—Tadpole in stage XIII, 20 days after implantation. *A*, the encapsulated thyroxin-cholesterol pellet appears dorsolateral to the right side of the diencephalon. Note the large glands above the pellet and in the adjacent supraorbital skin. Few glands appear on the left side. Loose molted skin appears above the right half of the head. ×40. *B*, bracketed section of *A*, enlarged to ×240.

PLATE I

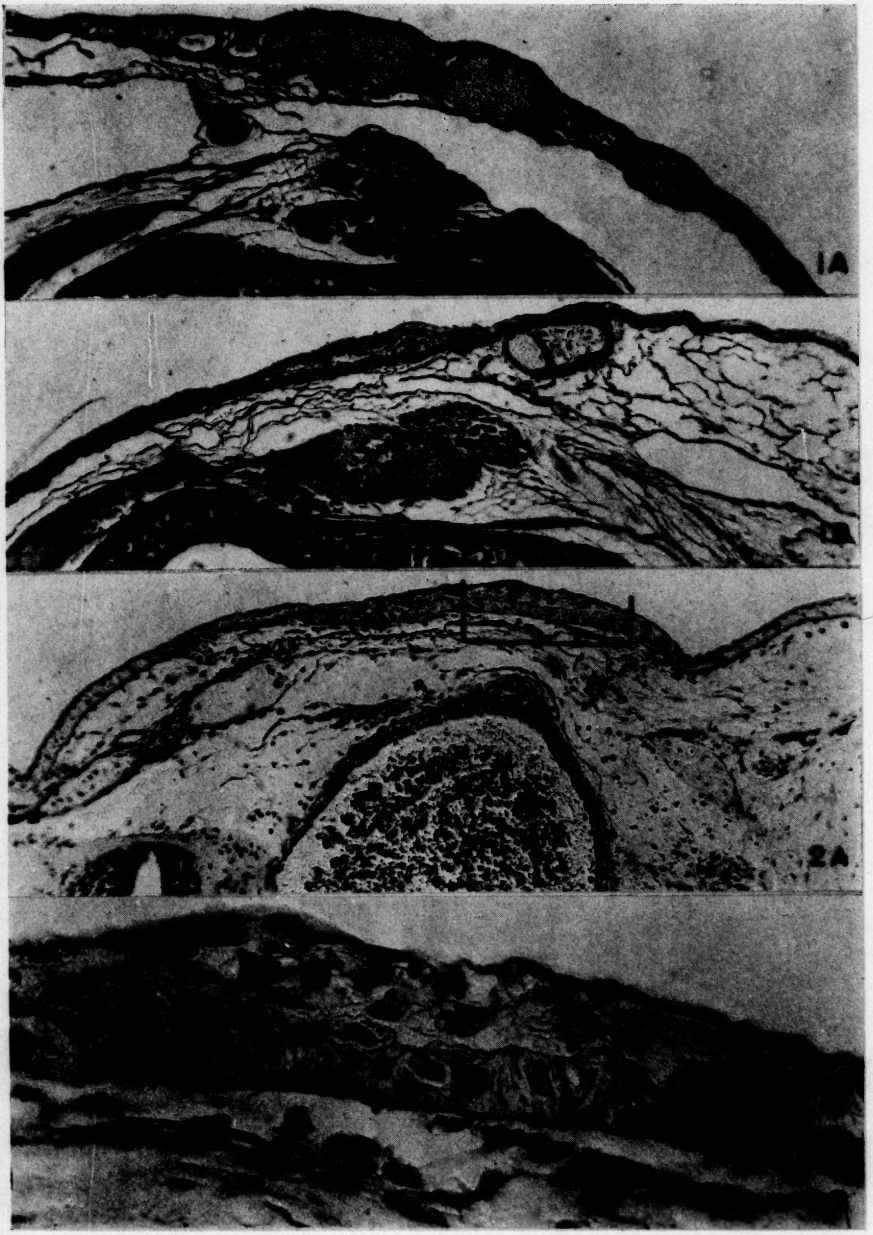
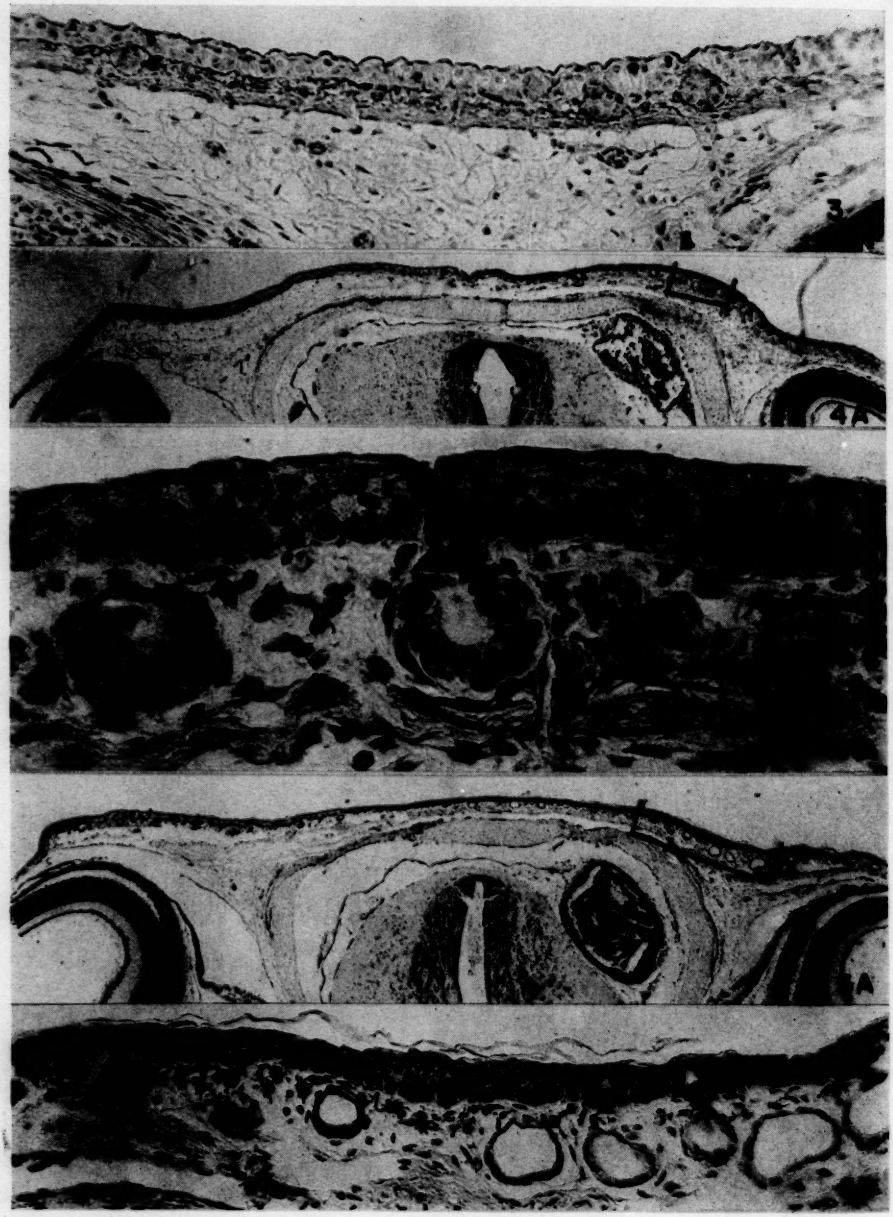


PLATE II



BIOCHEMICAL CHANGES DURING THE EMBRYONIC DEVELOPMENT OF THE JAPANESE BEETLE (POPILLIA JAPONICA NEWMAN)[1, 2]

FRED ROTHSTEIN
Department of Biology, University College, New York University

INVESTIGATIONS of the chemical aspects of insect embryology have been very sparse and limited in scope. Tichomiroff (1885) observed a decrease in fat, glycogen, and protein during the postdiapause development of the silkworm, *Bombyx mori*. These observations were confirmed by Farkas (1903), Vaney and Conte (1911), Pigorini (1923), Tirelli (1931), and Minoya (1937). Farkas also showed that the total nitrogen content remained constant throughout embryonic life and that, in agreement with Luciani and Piutti (1888), there is a gradual increase in CO_2 evolution following the termination of hibernation. The little work done on other species agrees with the reported tendency for fat and glycogen, serving as the sources of energy, to decrease during embryonic development. Weinland (1907), working with the blowfly, *Calliphora vomitaria*, found that the embryo consumes a large amount of fat; and Slifer (1930, 1932) reported that 54.3 per cent of the initial fatty-acid content of the egg of the grasshopper, *Melanoplus differentialis*, is lost during the developmental period. Hill (1943) confirmed Slifer's observations and concluded that fat is the main source of energy in the postdiapause development of the *Melanoplus* egg. Rudolfs (1926) observed the same reduction trend in the fat content of the eggs of the tent caterpillar, *Malacosoma americana*, and Busnel (1937) for those of the potato beetle, *Leptinotarsa decemlineata*. Rudolfs also recorded a decrease in glycogen during the development of the eggs of the tent caterpillar.

Fink (1925) examined the respiratory exchange of developing eggs of ten species of insects, employing manometric and colorimetric techniques. In each case there was a preliminary "formative period" during which the respiratory rate was very low, followed by a rapid increase in respiration, which continued until eclosion. In most forms the formative period is brief, not more than a day, but in the Japanese beetle, *Popillia japonica*, it lasts more than 6 days at temperatures between 21° and 24° C., after which there is a sharp rise in respiration. The significance of the formative period is unknown. Melvin (1928) determined the oxygen consumption of the eggs of four species of insects and also observed a formative period, followed by a steady rise in oxygen consumption. Bodine (1932), employing the eggs of *M. differentialis*, reported a rise in oxygen uptake during the postdiapause period. Boell (1935) found that the respiratory quotient of *Melanoplus* eggs during this period is 0.7. This value has been interpreted as indicating the use of fat, as shown by Slifer (1930, 1932).

[1] Submitted in partial fulfilment of the requirements for the degree of Master of Science at New York University. Present address: Biology Department, Massachusetts Institute of Technology, Cambridge, Mass.

[2] The author wishes to express sincerest gratitude for the stimulation, interest, and critical guidance of Dr. Daniel Ludwig. Thanks are also due to Dr. Milan J. Kopac for many valuable suggestions.

With respect to water metabolism, Bodine (1929) was the first to show by direct measurements that the water content of orthopteran eggs increases during development. These observations were confirmed by Roonwal (1936) for those of the orthopteran *Locusta migratoria*. Ludwig (1932) found that the development of the Japanese beetle egg was dependent on the absorption of water; water content at 20° C. increasing from 49.0 per cent in the newly laid egg to 84.0 per cent at the end of the embryonic period. However, not all insect eggs absorb water during development. Ludwig and Anderson (1942) showed that the eggs of four species of saturniid moths were able to develop at relative humidities ranging from 0 to 100 per cent. At the lower humidities, development was associated with a steady decrease in weight, the resulting larvae having a reduced water content.

This brief review indicates the need for a comprehensive and integrated investigation of the biochemistry of insect embryonic development. Accordingly, the following study of the biochemical changes which occur during the embryonic period of the Japanese beetle was initiated. This study includes determinations of water, nitrogen, glucose, glycogen, and free and bound lipid.

MATERIAL AND METHODS

The eggs used in these experiments were collected in the laboratory from beetles gathered in the field. Eggs were collected each morning, so that, when obtained, they were always less than 24 hours old. They were placed, one hundred to a box, in 1-ounce metal salve boxes containing moist soil and allowed to develop in an incubator at 30° C. At this temperature the embryonic period lasts 8 days. For each day of this period the desired number of eggs was weighed and then stored under vacuum desiccation until needed. Material retained under these conditions showed no difference from those analyzed immediately. The various analyses were conducted for each day of the embryonic period.

Water content was determined by evaporation in a vacuum. It was calculated from the original weight readings and those made after complete desiccation.

Nitrogen determinations were made on groups of 15 eggs by the micro-Kjeldahl procedure (Niederl and Niederl, 1938, pp. 51–59) as modified by Wagner (1940). Glycogen determinations were made on groups of 30 eggs by the method of Pflüger as modified by Good, Kramer, and Somogyi (1933). Analyses for reducing substances, expressed as glucose, were made by the Hagedorn and Jensen procedure as modified by Ludwig and Rothstein (1949).

Lipid determinations were made by both ether and ether-alcohol extraction on the same group of eggs in a Soxhlet apparatus. The Soxhlet flask was thoroughly dried over $CaCl_2$, weighed, and filled with the appropriate solvent. One hundred dried eggs were used for each extraction. They were pulverized with purified sand (see Bloor, 1929, p. 282) and transferred to the Soxhlet thimble. The eggs were first extracted with filtered anesthesia ether for a period of 8 hours. They were then extracted for the same period of time, employing another dried and tared Soxhlet flask, with ether-alcohol (3 parts of 95 per cent ethyl alcohol to 1 part ether). The solvents were evaporated and the flasks dried to constant weight. The differences between the final and initial weights of the flasks are the milligrams of ether-soluble lipid, expressed as free lipid, and ether–alcohol-soluble lipid, expressed as bound lipid, respectively.

OBSERVATIONS

The changes in the weight and water content which occur during the development of the Japanese beetle egg at 30° C. are shown in Table 1. The egg increases from an average initial weight of 0.83 mg. to 2.45 mg. in the 7-day egg. The increase in weight occurs very rapidly during the first 4 days, then more slowly until the sixth day, after which the weight remains approximately constant. Upon hatching, there is an average loss of weight equaling 0.54 mg. Corresponding to these weight changes, there is an increase in water content from approximately 50 per cent in the newly laid egg to 85 per cent in the egg just before hatching. The data contained in Table 1 show that the changes in weight are the result of changes in water content. This relationship is also shown in Figure 1.

Table 2 contains the results of the nitrogen determinations. There is no change in the total nitrogen content of the egg during the embryonic period, the value remaining at approximately 0.036 mg. per egg. Because of the increase in weight which occurs during this period, the per cent of nitrogen decreases. Upon hatching, there is a loss of weight, resulting from the shedding of the chorion and a loss of water, which is reflected by an increase in the percentage nitrogen value. The constancy in the amount of nitrogen is the control with which the variables may be compared. Therefore, the results of the other chemical determinations are expressed as percentages of nitrogen.

Variations in the concentration of glu-

TABLE 1

CHANGES IN WEIGHT AND WATER CONTENT DURING EMBRYONIC DEVELOPMENT

Age (Days)	No. of Individuals	Av. Wet Weight (Mg.)	Per Cent Water	Increase in Weight (Mg.)	Change in Water (Mg.)
Just laid	1,465	0.83±0.004*	49.97	.....	.....
1	1,465	1.02±0.015	60.03	+0.21	+0.20
2	1,715	1.85±0.018	79.11	+0.83	+0.85
3	1,465	2.25±0.016	81.83	+0.40	+0.38
4	1,315	2.30±0.012	83.06	+0.05	+0.06
5	1,315	2.41±0.012	83.84	+0.11	+0.11
6	1,315	2.48±0.013	84.33	+0.07	+0.07
7	1,315	2.45±0.008	84.68	−0.03	−0.02
Larva (just hatched)	1,315	1.91±0.015	81.58	−0.53	−0.52

* Probable error.

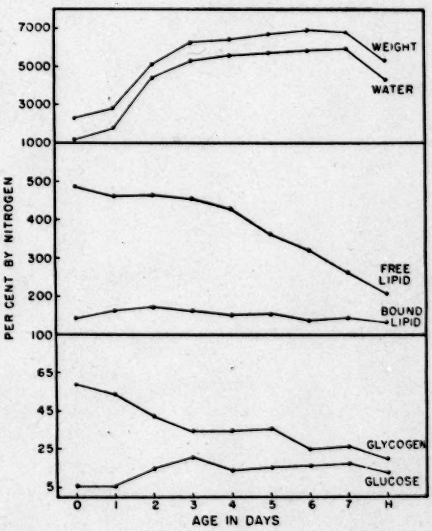


FIG. 1.—Changes in weight, water content, free and bound lipid, glycogen, and glucose during embryonic development. Values are plotted against the concentration of nitrogen. Abbreviations: *O*, newly laid egg; *H*, newly hatched larva.

cose are shown in Table 3 and Figure 1. Glucose content remains constant during the first 2 days. It then increases for the next 2 days, reaching a maximal value of 0.74 mg/100 eggs. The differences in concentration between 1-day, 2-day, and 3-day eggs are significant. There is a significant decrease in glucose between the 3-day and the 4-day eggs. This increase is followed by a gradual rise in glucose concentration, which continues until the end of the embryonic period. This rise is so gradual that differences between the values obtained for any two consecutive days are not significant. However, the differences between those obtained for the 4-day eggs and those for the 6-day or 7-day eggs are significant. There is a significant decrease in concentration at the time of hatching. The data presented indicate an over-all increase in glucose during embryogenesis. Because of the increase in weight which occurs during this period, the percentage values by weight for glucose remain approximately constant. However, when the percentage values are calculated with respect to the nitrogen concentration (last column of Table 3), the increase in glucose is clearly shown.

The values obtained in the glycogen analyses are presented in Table 4 and Figure 1. The glycogen content decreases from the newly laid egg to the 3-day egg.

TABLE 2

TOTAL NITROGEN CONTENT OF THE JAPANESE BEETLE DURING EMBRYOGENESIS

Age (Days)	No. of Individuals	Av. Weight (Mg.)	Av. Weight of Nitrogen (Mg.)	Per Cent Nitrogen
Just laid	150	0.79	0.036±0.00023	4.60
1	150	0.88	0.037±0.00031	4.18
2	150	1.66	0.036±0.00038	2.19
3	150	2.13	0.036±0.00023	1.68
4	150	2.19	0.035±0.00040	1.62
5	150	2.29	0.035±0.00034	1.52
6	150	2.25	0.035±0.00044	1.56
7	150	2.47	0.037±0.00036	1.48
Larva (just hatched)	150	1.91	0.035±0.00057	1.87

TABLE 3

GLUCOSE CONTENT OF THE JAPANESE BEETLE DURING EMBRYOGENESIS

Age (Days)	No. of Individuals	Av. Weight (Mg.)	Glucose per 100 Individuals (Mg.)	Per Cent by Weight	Per Cent by Nitrogen
Just laid	600	0.83	0.20±0.009	0.25	5.55
1	600	0.97	0.20±0.006	0.21	5.55
2	750	1.92	0.52±0.026	0.28	14.44
3	600	2.31	0.74±0.034	0.32	20.56
4	450	2.39	0.50±0.015	0.21	13.89
5	450	2.48	0.54±0.021	0.22	15.00
6	450	2.55	0.58±0.017	0.23	16.11
7	450	2.43	0.61±0.018	0.26	16.94
Larva (just hatched)	450	1.95	0.45±0.028	0.23	12.50

The decrease for each day is statistically significant. The glycogen content then remains constant until the 5-day egg, when it again decreases. The total loss of glycogen is approximately 66 per cent of the available supply. The change in glycogen content during embryogenesis is shown in the table by both percentage values, weight and nitrogen. However, because of the increase in weight, the percentages calculated by weight indicate a much greater loss of glycogen than actually occurs.

The results of the lipid determinations are given in Table 5 and Figure 1; there is a relatively large amount of lipid in the Japanese beetle egg. Because each determination was made on only four groups of 100 eggs, no statistical treatment was attempted. The total lipid (free lipid plus bound lipid) content remains constant until the 3-day egg. Beginning with the fourth day of embryonic development, there is a marked decrease in free lipid, which continues until the time of hatching. The bound lipid, on the other hand, remains essentially constant throughout the embryonic period. The over-all loss of the ether-soluble material is equivalent to 25 per cent of the initial dry weight of the egg and to 58 per cent of the initial amount of free lipid.

TABLE 4

GLYCOGEN CONTENT OF THE JAPANESE BEETLE DURING EMBRYOGENESIS

Age (Days)	No. of Individuals	Av. Weight (Mg.)	Glycogen per 100 Individuals (Mg.)	Per Cent by Weight	Per Cent by Nitrogen
Just laid	450	0.86	2.09±0.02	2.43	58.4
1	450	1.17	1.93±0.03	1.65	53.6
2	450	1.79	1.53±0.03	0.86	42.6
3	450	2.24	1.24±0.04	0.56	34.4
4	450	2.36	1.24±0.05	0.53	34.4
5	450	2.43	1.26±0.05	0.52	35.6
6	450	2.49	0.86±0.05	0.35	24.6
7	450	2.44	0.93±0.02	0.39	25.9
Larva (just hatched)	450	1.90	0.71±0.03	0.37	19.7

TABLE 5

LIPID CONTENT OF THE JAPANESE BEETLE DURING EMBRYOGENESIS

Age (Days)	No. of Individuals	Av. Weight (Mg.)	Free Lipid			Bound Lipid		
			Per 100 Individuals (Mg.)	Per Cent by Weight	Per Cent by Nitrogen	Per 100 Individuals (Mg.)	Per Cent by Weight	Per Cent by Nitrogen
Just laid	400	0.85	17.5	20.58	486.11	5.1	6.00	141.67
1	400	1.07	16.6	15.51	461.11	5.8	5.42	161.11
2	400	2.02	16.7	8.27	463.89	6.2	3.07	172.22
3	400	2.31	16.4	7.10	455.56	5.8	2.51	161.11
4	400	2.41	15.4	6.39	427.78	5.4	2.24	150.00
5	400	2.43	13.0	5.25	361.11	5.5	2.26	152.78
6	400	2.46	11.5	4.67	319.44	4.9	1.99	136.11
7	400	2.51	9.4	3.75	261.11	5.2	2.07	142.08
Larva (just hatched)	400	1.90	7.4	3.89	205.56	4.9	2.53	133.33

DISCUSSION

The changes in weight and water content of the Japanese beetle egg reported here are similar to those found by Ludwig (1932) for the egg of this insect. He allowed the eggs to develop at 20° and 25° C., and the rapid increase in weight and associated uptake of water continued approximately 11 and 6 days, respectively, as compared with 4 days in the present experiments at 30° C. The increase in water content observed during embryonic development agrees with the work of Bodine (1929) and Roonwal (1936), both studying orthopteran eggs. It thus appears that the newly laid eggs of some species of insects are in a state of partial dehydration and, in order to survive, must have water with which to carry on the metabolic processes responsible for development.

Oviparous forms lay eggs, which, with the exception of gaseous and water exchange, are "metabolite-closed" or nonfeeding systems. It is therefore to be expected that all the essentials, except oxygen and water, needed for embryonic development are present in the egg. This condition would lead to the assumption that there is an optimal utilization of all available material. It is not surprising, therefore, that the eggs of the Japanese beetle do not lose nitrogen during development. Similar observations were made by Anderson (1948) for the pupae of this insect, which are also metabolite-closed or nonfeeding systems.

The decrease in glycogen reported here is in agreement with the results of previous workers. In addition, the time of its utilization also agrees with that found in other metabolite-closed systems. It is observed that carbohydrate is initially employed, followed by lipid, as the energy source. This is the same sequence as was found during the pupal stage of the Japanese beetle (Ludwig and Rothstein, 1949) and during starvation in the grasshopper, *Chortophaga viridifasciata* (Ludwig, 1950), both nonfeeding systems. The increase in concentration of reducing compounds during the early part of embryogenesis occurs at the same time that glycogen decreases in amount. This observation suggests that glycogen is the material from which the reducing compounds are formed. However, glycogen breakdown occurs at a greater rate than is required for the production of reducing compounds. Thus glycogen appears to serve as the source of energy during the first 3 days of embryonic development. Preliminary observations on oxygen consumption indicate that at 30° C. there is also a low, constant uptake of oxygen during the first 3 days (unpublished data). This period, called the "formative period" by Fink (1925), is thus coincident with the drop in glycogen content. The second phase of glycogen utilization occurs near the end of embryonic development at a time when free lipid is being rapidly utilized.

A study of Tables 4 and 5 shows that free lipid is the main source of energy during later embryogenesis and, in fact, throughout the entire period considered as a whole, since its over-all loss is much greater than that of glycogen. The loss of glycogen amounts to only 1.38, while that of free lipid reaches 10.1 mg/100 eggs. This observation is in complete agreement with other work. All available data, as summarized by Needham (1931, 1942), point to free lipid as the main source of energy during the development of the eggs of forms belonging to many classes and phyla. The amount of free lipid used during the development of the Japanese beetle egg (approximately 58 per cent) compares favorably with the decrease in fatty acid (54.3 per cent) reported by Slifer (1930, 1932) for the egg of the grasshopper, *M. differentialis*. The

rapid utilization of free lipid, beginning with the 4-day egg, corresponds with the period of high oxygen utilization. Thus it would seem as if this latter period of embryogenesis is one of high energy turnover and reflects an interval of intense synthetic and organizational activity.

The present work lays the basis for more specific investigations on the biochemistry of embryonic development in the Japanese beetle. A study of the fractionation of nitrogenous compounds and the variation in the concentration of nitrogen in each fraction is now being completed and should add substantially to the understanding of the developmental process.

SUMMARY

1. The Japanese beetle egg, at 30° C., increases from an average initial weight of 0.83 mg. to 2.45 mg. at the end of the embryonic period. This increase in weight is associated with the absorption of water, the water content increasing from 50.0 per cent in the newly laid egg to 84.7 per cent in the egg at the end of the embryonic period. The changes in weight are the result of changes in water content.

2. The total nitrogen content remains constant during embryogenesis, at an average value of 0.036 mg. per egg.

3. The glucose content increases during embryogenesis. It has an average value of 0.0020 mg. in the newly laid egg. Following the second day, the glucose content increases rapidly, reaching a maximal value of 0.0074 mg. in the 3-day egg. There is then a decrease to 0.0050 mg. in the 4-day egg, followed by a gradual but significant increase during the remainder of the embryonic period.

4. Glycogen decreases rapidly during the early part of embryogenesis from an initial value of 0.021–0.012 mg. in the 3-day egg. A second decrease in glycogen occurs near the end of the embryonic period. Glycogen appears to furnish the material from which the glucose is formed. In addition, it serves as the main source of energy during the early part of embryogenesis, when the rate of oxygen consumption is low.

5. Total lipid remains constant during the early part of the embryonic period. Beginning with the fourth day, there is a marked decrease in free lipid, which continues until the time of hatching. The total loss of free lipid amounts to approximately 58 per cent of the available supply. It furnishes the main source of energy during embryogenesis. Bound lipid, on the other hand, remains constant throughout the embryonic period.

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A LABORATORY STUDY OF INTRASPECIES AND INTERSPECIES COMPETITION IN DAPHNIA PULICARIA (FORBES) AND SIMOCEPHALUS VETULUS O. F. MÜLLER[1]

PETER W. FRANK[2]

Department of Zoölogy, University of Chicago, Chicago, Illinois

BECAUSE of their significance with respect to the temporal and spatial abundance of organisms, studies of interspecific competition have become one of the foci of ecological research. While the mixed-species problem seems to have been appreciated by several earlier naturalists, it was first clearly defined by Darwin in *The Origin of Species*. He considered competition to be one of a number of checks to an un-

[1] Dr. Thomas Park contributed many valuable suggestions during the course of this study and in the preparation of the manuscript. Marian Burton Frank discussed segments of the data with the author at various times and read the manuscript critically. Dr. J. L. Brooks, Yale University, kindly identified the *Daphnia*.

[2] Present address: Osborn Zoölogical Laboratory, Yale University, New Haven, Connecticut.

bounded increase of animals and reached many of the basic conclusions regarding the causes and effects of competition. Despite the fact that a field for investigation was thus clearly outlined, little evidence seems to have been accumulated until Volterra (1926, 1931) and Lotka (1932), by constructing a mathematical theory of interactions between species, gave renewed impetus to experimentation. Gause (1932, 1935), working mainly with yeasts and ciliates, was able to confirm in the laboratory some of the results predicted from mathematical models. Since that time there have been an increasing number of investigations both of laboratory and, to a less extent, of natural populations. Crombie (1947), in a comprehensive review, has presented an extensive bibliography of pertinent research.

Competition, according to Clements and Shelford (1939), consists of the demand, typically at the same time, of more than one organism for the same environmental resources in excess of immediate supply. This definition needs to be interpreted rather freely to be sufficiently broad to include such phenomena as competition arising from the accumulation of metabolites. According to Crombie (1947), a competitive advantage may be conferred upon one group of organisms over others by an intrinsically higher birth rate, lower death rate, or higher proportion of females or by a greater power of interference with the characteristics of reproduction and survival of the other groups of organisms. "Interference" in this context denotes competitive pressures arising specifically from the presence of two or more competing populations. The terms "intrinsic" and "interference" will be used throughout this discussion to distinguish between single-species effects and those concurrent only with cohabitation of two species. It is likely that, in any particular instance, both intrinsic factors and interference affect the end-result, although all the components do not necessarily act in the same direction or at the same time. Which of two species is favored depends on the resultant, and changes in the environment, therefore, may reverse the position of advantage of one species over another. This is elegantly demonstrated by an extensive laboratory study of two species of flour beetles, *Tribolium* (Park, 1948).

Reasonable agreement seems to exist on the probable consequences of interspecific competition. The following generalizations, stated or implied in Crombie's review (1947) or in the more general discussions by Allee, Emerson, Park, Park, and Schmidt (1949) and by Hutchinson and Deevey (1949), seem to be generally accepted on logical grounds, although critical evidence concerning many of them is still lacking. The outcomes of interspecific competition may vary. With identical needs and habits, all but one of a number of species in competition with one another are eliminated. With similar needs and habits, the result may be the same but depends on the degree of similarity and the complexity of the environment. If the latter is sufficiently diverse in space, several species may survive in different habitats, with competition occurring only where these abut and where the species are about equally favored. With an environment that is variable only with respect to time, one species may be favored over others for short, recurring periods, and again several species may survive, at least for an appreciable time span, undergoing forced fluctuations in density.

Natural environments are variable both in time and in space. Therefore, competitive relations become exceedingly difficult to evaluate, quite aside

from the technical problems involved in measuring the parameters necessary to assess the intensity of the various factors involved. For this reason it is understandable that few well-substantiated field analyses of competition between species have been reported. Ullyett's (1950) monograph on competition in sheep blowflies, in which both field and laboratory data are discussed, demonstrates the complexities inherent in such a study, even in a relatively simple environment. Besides such direct attempts to establish the effects of interspecies competition in natural populations, efforts have been made to demonstrate the role of competition by comparing observed distributions of certain species with those that might have been expected in the absence of competitive pressures. This a posteriori approach has met with considerable success (Lack, 1947) but has sometimes presented difficulties of interpretation (Elton, 1946; Williams, 1947, 1951).

The most detailed direct evidence regarding interspecies competition has resulted largely from laboratory investigations. The organisms that have been chosen for such studies cover a wide range of invertebrates; acellular aquatic organisms and graminivorous insects have, however, been favored, since they are eminently suitable for laboratory culture under conditions closely simulating those of their natural habitats.

Daphnids have a fairly long history as objects of experimental research, mainly owing to the interesting phenomenon of cyclomorphosis (Wesenberg-Lund, 1908) and to their peculiar modes of reproduction (Weismann, 1876). Quantitative studies on strictly populational aspects of Cladocera are not too numerous. Aside from some estimates of natural population density (Eddy, 1934; Ward, 1940; Pennak, 1946), a number of laboratory investigations present data on reproductive rates and survival of several species of *Daphnia*. In this regard *D. magna* has been analyzed by MacArthur and Baillie (1929*a*) and by Pratt (1943); *D. longispina* by Ingle, Wood, and Banta (1937) and by Dunham (1938); and *D. pulex* by Anderson, Lumer, and Zupancic (1937). Of these, the only study primarily concerned with population growth-form is that of Pratt. His analysis of population development at two temperatures provides an excellent example of the subtle factors that may cause major differences in growth-form of a species population. Slobodkin (1951), working with *D. obtusa*, has made an attempt to provide a mathematical model predicting fluctuations of the type observed by Pratt and found by Slobodkin to be dependent on the quantity of food available. A series of papers by Terao and Tanaka (1928*a*, *b*, *c*, 1930) completes the list of such investigations that have come to the attention of the author.

The present paper attempts to describe the operation and result of competition between laboratory populations of *D. pulicaria* (Forbes) and *Simocephalus vetulus* O. F. Müller. These two species are commonly found in the same pools, ponds, and lakes. In the larger bodies of water, *Daphnia* tends to be pelagic, while *Simocephalus* is exclusively littoral (Wagler, 1937), so that competition between them, if it occurs at all, may be presumed to be minimal. It seems likely, however, that in small pools, in which the species are more restricted in habitat, while at the same time frequently attaining high densities, competition may at times become pronounced. As will be shown, under certain laboratory conditions when the habitats of the two species become nearly indistinguishable, the effects of competition become quite severe.

The basic experiments to be discussed

describe the growth-form of mixed-species populations. Appropriate controls of single-species populations were also observed in order to determine which of the phenomena resulted from interspecies competition. Several assays of mortality and natality of both species, under a variety of conditions related to density, make possible a partial analysis of the component factors of the competitive situation. In a later section these experiments are discussed in reverse order, to permit more logical presentation.

MATERIAL AND METHODS

Daphnids possess a number of characteristics which make them favorable for certain types of population study. They can be made to reproduce exclusively by diploid parthenogenesis, so that genetically identical animals are readily available. Their life-cycle is relatively short. Since the parthenogenetic eggs develop in the mother's brood pouch to the neonate stage, which closely resembles the adult except in size, several complexities inherent in the life-cycles of many arthropods are avoided. Certain difficulties are encountered, however. Feasible laboratory environments are not strictly comparable to those in which the forms normally live. Although daphnids are readily cultured, contamination of the medium by various microörganisms is difficult to prevent. Certain species of Cladocera are highly labile so far as production of males is concerned, for reasons that are still partially obscure with respect to both causal agents and genetic mechanism (Banta, Wood, Brown, and Ingle, 1939). However, the species here under investigation produced a maximum of less than 2 per cent males, and usually the experimental populations consisted entirely of females.

Both species were originally obtained from a pool near one of the large garbage-disposal stations for Chicago. When the present work was initiated, they had been mass-cultured together in large laboratory tanks for about 2 years. One vigorous clone of each species was selected. Other individuals were never introduced into the laboratory during the course of the research. Deliberate frequent reduction of stock cultures to one animal per species served as an additional precaution against contamination by different strains.

Throughout the study the daphnids were fed on a suspension of an aquatic species of *Chlorococcum*. This alga is easily handled and reproduces rapidly in the laboratory; it has the drawback of reproducing asexually exclusively by means of zoöspores, so that the algal population consists of a variable proportion of cells of two sizes, which may possibly differ also in chemical constitution. An attempt was made to reduce this variability to a minimum by using only subcultures between 8 and 16 days of age. A pure culture of algae was prepared by a combination of methods recommended by Pringsheim (1946). Excellent growth was obtained on a 1.5 per cent agar medium containing 0.4 per cent NH_4NO_3, 0.3 per cent KH_2PO_4, 0.1 per cent $MgSO_4 \cdot 7H_2O$, and 0.0001 per cent $FeSO_4$ in charcoal-filtered tap water. In preparing the medium for the daphnids, the algae were removed from the agar and suspended in a small quantity of water, and the algal cells were separated by means of a motor stirrer. This suspension was then added to a larger amount of charcoal-filtered water, and estimations of algal cells were made with a hemocytometer. The total volume of the medium was adjusted to a density of approximately 1.5 million algal cells per cubic centimeter of water. Because the algae settled slowly, this concentration is not too exact.

All experimental animals were maintained at a mean temperature of 21°.1 C., with a total temperature range of less than 1°, except for a few brief periods of power failure, when temperatures went as high as 25° C. Constant light of average intensity of 220 foot-candles at the level of the cultures was maintained by a large fluorescent fixture. Culture bottles used in most of the experiments contained 165 cc. and had a diameter of 5.5 cm., making the water about 9.5 cm. deep. In two experiments, bottles of half the volume, 4.2 cm. in diameter, were employed (called hereafter "half-sized bottles").

Total counts of all animals were made at 2-day intervals throughout. The medium was completely changed at the same time; debris, including ephippia, was discarded. A few simple techniques were devised, by means of which it was feasible to count about fifteen thousand animals per day. In general, the method of counting had no injurious effects on the animals. A bolting-cloth sieve was used to remove neonates from adults in the natality experiments. In the experiments on population growth-form, a somewhat subjective separation of the daphnids into adults and young was made on a visual basis, instead. While this distinction probably has some validity, it is felt that some differences in judgment occurred, if not from one census to the next, at least over longer periods of time.

To determine the precision of the census methods employed, populations of both species, each containing exactly 500 individuals, and consisting of 340 adults and 160 young, were counted ten times. For *Simocephalus* the observed means and standard errors were 339.8 ± 1.6 adults, 159.3 ± 1.4 young, with a total of 499.2 ± 2.1; for *Daphnia* the respective figures were 341.1 ± 1.7, 158.9 ± 1.6, and 500.0 ± 2.5. During most of the experiments the actual precision attained was probably higher than these figures would indicate, since the counts were purposely made less exact than was usual. It is possible that, for the initial stages of the program, this estimate of reliability is somewhat too good.

Mixed-species experiments were simplified technically by the fact that *Daphnia* and *Simocephalus* are readily distinguished with the unaided eye; with some practice, even neonates of the species can be separated. This is brought about not so much by morphological differences as by the behavior characteristics of the two genera. The adaptations for clinging to objects, which *Simocephalus*[3] possesses and *Daphnia*[3] lacks, imply this behavioral difference. *Simocephalus* usually is found attached dorsally to the walls of the container; *Daphnia*, on the other hand, is continuously in motion. In addition, *S.* swims at a fairly even rate, while *D.* moves by means of discrete jerks, from which its common name derives. Differences in color and shape are helpful as subsidiary methods of distinction.

Certain more specific descriptions of technique and design are necessary for different groups of experiments. These methods become more meaningful in connection with the experimental results, and they are therefore interspersed through the following sections.

NATALITY

Before examining the effect of population density, a brief outline of the reproductive behavior of uncrowded animals under standard conditions seems advisable. Neonates of both species usually become reproductively active after the completion of four instars. Young are

[3] Sometimes hereafter called "*S.*" and "*D.*," respectively.

then produced in broods that escape from the mother's brood chamber at ecdysis. The average time between successive molts increases with advancing age. This period is appreciably shorter in *Daphnia*, with a minimum of slightly less than 2 days, while in *Simocephalus* the period is at least $2\frac{1}{2}$ days in length. Brood size in *Daphnia* rises from between six and twelve at the first adult instar to above thirty at the maximum near the middle of the reproductive period, and then declines slowly. During the last few instars no reproduction may occur. In *Simocephalus* the pattern is quite similar, except that maximum brood size is exceptionally as high as fifty animals. These results are in good agreement with those reported for other daphnids (Banta *et al.*, 1939).

The above conclusions were reached from forty replicate records of single individuals of each species. For the study of both natality and mortality at higher densities, a somewhat more complex technique was required, since the ingenious and more precise method of Pratt (1943) could not be adapted. Originally, four replicate cultures were prepared for each density. Only the first of these was accurately censused at 2-day intervals. All young born during the interval were removed from all replicates. The number of adults that had died in replicate 1 was replaced by animals from replicate 2. This culture was in turn restored to approximately initial density by adding twice the number removed on the assumption that its death rate equaled that of replicate 1. Replicate 3, thus left with too few individuals, was in turn restored from replicate 4. This procedure was intended to keep the assay culture at constant density and was continued until replicate 2 no longer had close to the original numbers, when it was felt that the inaccuracies resulting from this transfer technique were too great to make further counts useful. It is possible by this procedure to obtain data on both natality and mortality. The data cannot be complete, however, since it is necessary to discontinue the experiments before all animals have died. There are two other shortcomings of results obtained by this method. First, during the initial days of the experiments all crowding is produced by immature individuals, and it is unlikely that their effect is equivalent to that of adults. Second, the transfer procedure results in the addition to replicate 1 of some animals which have not lived at the designated density for their entire life-span. These individuals are indistinguishable from the rest and have to be regarded with them in calculating birth and death rates. Thus the theoretical densities are not completely realized biologically; the error becomes appreciable particularly during the first and last days of an experiment and is probably greater at the higher densities. Some caution is therefore necessary in the interpretation of these data. They certainly cannot be used to obtain adequate estimates of theoretical asymptotic densities. Furthermore, in the absence of counted replicates, statistics of reliability cannot be determined. Replication has not usually been attempted in studies of this sort; the laboriousness of these experiments made it impracticable in the present instance. Some measure of validity is achieved because of the relatively large number of individuals in any one cohort.

Pratt (1943) reported that, as in a number of quite diverse organisms studied in this regard, reproductivity in *D. magna* varies inversely with density. In his study, reproductivity dropped sharply with relatively small increases in density and, as more extreme crowding was achieved, leveled off to approach no

natality at the highest density. Figure 1 graphs the effect of density on the reproductive rates of *D. pulicaria* and *S. vetulus* (note that the abscissa is logarithmic). Despite the reservations previously discussed, certain trends are so clear that considerable meaning can be attached to the graph for purposes of generalized statements.

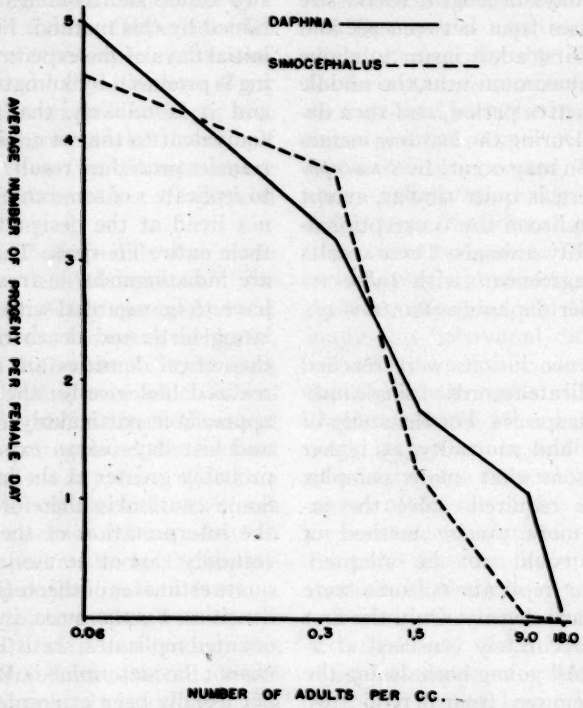

FIG. 1.—Effect of density on natality of single-species cohorts of *Daphnia* and *Simocephalus*. Parental density, 0.15/cc.

At least two conclusions can be drawn with reasonable certainty. First, the natality of both species declines rapidly as density increases, and it follows the pattern observed by Pratt (1943). The second statement involves a comparison of the birth rates of the two species. The ratio of natality of one species to that of the other is easily obtained from the figure. From this it is apparent that the reproductive rate of both species is at least grossly similar to a density of about 1.5 animals/cc; at higher densities the natality of *Daphnia* is a multiple of that of *Simocephalus*, becoming approximately sixteen times as great at a density of 9/cc. As still higher densities are reached, the natality of both species approaches zero, and this striking difference again disappears. Additional data are needed before the differences in reproductive rate observed at the three lowest densities can be taken seriously.

The above description has referred to females whose mothers had lived at relatively low densities (0.15/cc). A subsidiary study of reproductive rates of animals whose mothers had been more severely crowded (9/cc, or 750 per half-

sized bottle) gave some indication that parental density has an influence on the natality of the next generation, thus adding another variable. The results are summarized in Table 1. Natality appears to be reduced as a result of crowding the previous generation; the decline seems to occur in both species, but at different rates. Although, at low parental density and an experimental density of 9/cc, the reproductive rate of *Daphnia* is considerably higher than that of *Simocephalus*, this difference is virtually canceled at the higher parental density. The explanation for the effect of parental density may lie in the fact that neonates from crowded mothers are smaller than those produced by uncrowded mothers. The young may be unable to compensate for this difference during their life-span, at least under the present conditions of culture. Substantiation of this hypothesis has not been attempted.

TABLE 1

THE EFFECT OF PARENTAL DENSITY ON OFFSPRING PRODUCED BY THE NEXT GENERATION

Parental Density (Per Cc.)	Density of Experimental Animals	Offspring Produced per Female Day	
		Daphnia	*Simocephalus*
0.15.....	0.3	3.2	3.7
	9.0	1.1	0.061
9.0.....	0.3	1.3	1.6
	9.0	0.092	0.055

An effort was made, however, to determine to what extent a similar lag in reproductivity existed in animals subjected to sudden changes in density. The results (Fig. 2) give indications for each species from eight censused cultures at a density of 0.6/cc, changed to a density of 9/cc on day 10, and of the reverse procedure. Age-specific reproductive rates for animals at constant densities of 0.6 and 9/cc are graphed as controls. Those animals which had been exposed to more severe crowding after a period at relatively low density exhibited a drop in reproductive rate by the next census; they did not, however, reach the lower rate of the controls that had lived continuously at the higher density until 6 days later. Conversely, crowded animals, when shifted to a low density, did not show an appreciable increase in natality for about 4 days but, by 10 days after the change, produced the same number of progeny as did control animals at the lower density. There is therefore a certain period of recovery from the effects of crowding, whatever their cause, during which the new conditions are not reflected by the criterion of natality. Similar lags have been observed by Pratt (1943) and by Slobodkin (1951).

Not only the separate reproductive behavior of the two species but also that resulting when they live together is significant in an analysis of competition between them. Two mixed-species experiments, at total densities of 1.5 and of 6 animals/cc, were performed. The actual proportions of the two species are given in Table 2. No a priori reason exists for assuming a one-to-one relationship between the amount of chemical or physical crowding produced by a certain number of one species and that produced by the same number of the other. The data presented show that in the present instance, despite the similarity in size of individuals, the two species are not equivalent in this respect. At the lower density there is little indication of this fact. Particularly in the case of *Daphnia*, natality is not markedly different when crowding is produced by *Simocephalus* than when crowding results from members of its own species; nor are the differences in reproductivity of *S.* at the lower density striking, vaguely indicating that *D.* inhibits the natality of *S.* less than *S.*

TABLE 2

NATALITY IN MIXED-SPECIES POPULATIONS

Total Density (Per Cc.)	Ratio *Daphnia*:*Simocephalus*	Number of Progeny per Female Day	
		Daphnia	*Simocephalus*
1.5.....	248: 2	1.9	1.5
	215: 35	1.9	1.3
	125:125	1.9	1.3
	35:215	2.1	1.0
	2:248	2.0	1.1
6.0.....	990: 10	0.11	0.25
	900:100	0.13	0.03
	500:500	0.14	0.04
	100:900	0.65	0.04
	10:990	0.78	0.05

does its own. At the higher density such differences become more marked. *Simocephalus* does not inhibit the natality of *D.* to the extent that *D.* does. This is demonstrated by the steady, although nonlinear, decrease in reproductive rate of *D.* as the relative number of *D.* increases. With *S.* the case is somewhat different. Up to a certain point, *D.* seems to inhibit *S.* about as much as *S.* does itself; when the proportion of *S.* becomes very small, however, the number of progeny per female increases markedly. This increase must be considered significant. The design of these experiments was similar to those dealing with single-

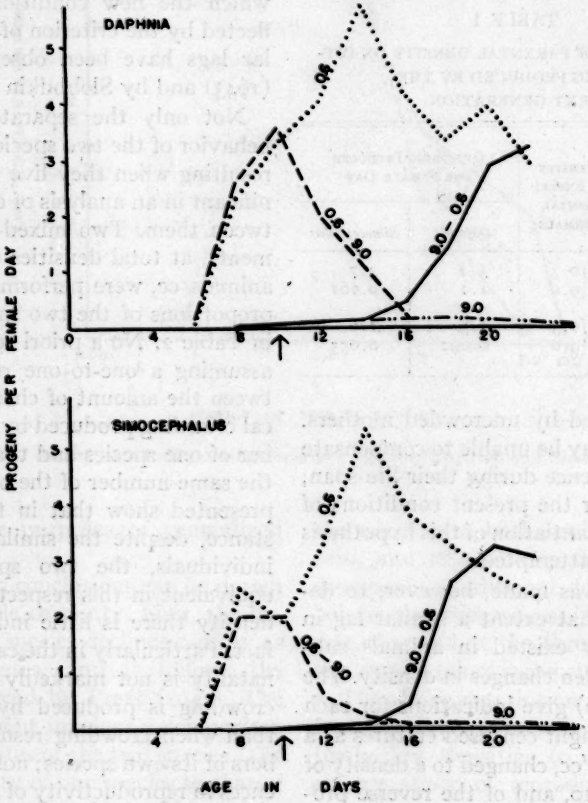

FIG. 2.—Effect of sudden density changes on natality. Parental density, 0.6/cc

species cohorts, and all the criticisms discussed previously apply. At a given total density, however, all populations could be followed for the same length of time in the present case, so that certain errors are reduced to a minimum. The number of *S.* involved is small; nevertheless, the increase in natality was consistent over a period of time and is so great that it cannot be easily accounted for on the basis of chance variation.

These results suggest a number of speculations. The relation between the relative crowding of the two species and their reproductivity seems to be nonlinear at the higher densities. The rather abrupt rise in natality of *S.* when it represents a low proportion of the total population is rather curious and is not easily explained on the assumption that the effect is produced directly by some type of chemical conditioning. The possibility that, when its numbers are quite low, *Simocephalus* is able, to some extent, to escape from the action of crowding, suggests itself. This likelihood will be more fully discussed below. The fact that this difference is not particularly noticeable at the lower total density may lead one to the conjecture that at least two factors limiting natality may exist under the present conditions. The most obvious conclusion from the data is that the reproductivity of *S.* in mixed-species cultures in the observed range of densities is considerably below that of *D.*, except when the former species constitutes only a small fraction of the total population.

In all the experiments discussed so far, changes in reproductive rates have been observed by employing the number of births as a measure. How the observed limitation in natality is accomplished presents an analytical problem of a different sort. It may result from the death of eggs in the brood pouch or from a limitation in the production of parthenogenetic eggs. The first possibility can account for only a small part of the differences observed at various densities. This can be stated with some assurance despite the absence of quantitative data. At the higher densities, the percentage of animals with eggs in the brood chamber was always low. The number of eggs observed in single individuals also tended to be less at the higher densities. It is therefore likely that differences in natality are associated primarily with lower parthenogenetic egg production. Another consideration must be added. Under present conditions, sexual eggs were nearly always infertile and never contributed to natality. These sexual eggs are also shed at ecdysis. For any single molt, sexual and parthenogenetic eggs are mutually exclusive. The more sexual eggs produced, therefore, the lower the natality will be. Table 3 shows the average proportion of females with ephippial eggs in single-species populations at different densities. It is quite apparent from these data that the production of ephippial eggs, while a major component of the factors decreasing natality with crowding, cannot be regarded as the only one operating. It could not decrease the reproductive rate sufficiently and does not vary inversely with natality. Furthermore, ephippial egg production seems to

TABLE 3

RATE OF SEXUAL EGG PRODUCTION AT SEVERAL DENSITIES

Density (Per Cc.)	Percentage of Adult Females with Ephippia	
	Daphnia	*Simocephalus*
0.3	0	0
1.5	7.4	0
9.0	10.3	9.5
18.0	0.63	1.6

be more readily induced in *D.* than in *S.*, as is shown at a density of 1.5 animals/cc.

Little is known about what other mechanism may limit natality in the present experiments. Fox, Gilchrist, and Phear (1951) have reported a positive correlation between natality, food intake, and the oxygen tension of the medium for a number of species of *Daphnia*. While there is some evidence that food was not a primary limiting factor in the present instance, a critical analysis of the limitations of the environment remains to be done.

MORTALITY

The types of data describing and analyzing mortality under a variety of circumstances closely parallel those considered in the preceding section. Likewise, they have the same shortcomings, i.e., lack of replication and necessarily premature discontinuance; but they again appear to demonstrate quite strikingly a number of characteristics, including certain species differences that can be expected to affect the performance of the two species in a competitive situation.

For single-species populations at a number of densities, certain basic data are summarized in the condensed survivorship data (Table 4). The design of the experiments involves an approximation of constant density of assay animals by means of the previously discussed

TABLE 4

SURVIVORSHIP AND DEATH RATE OF SINGLE-SPECIES COHORTS

Density (Per Cc.)	x (Days)	*Daphnia*		*Simocephalus*	
		l_x	1,000 q_x (Per Day)	l_x	1,000 q_x (Per Day)
0.3	0–8	50	0	50	0
	8–16	50	0	50	0
	16–24	50	12.0	50	0
	24–32	45.2	113.0	50	55.5
	32–40	0*	.....	27.8	125.0
1.5	0–8	250	1.0	250	0
	8–16	248	3.0	250	4.0
	16–24	242.0	3.9	242.1	32.5
	24–32	234.4	11.6	179.1	39.9
	32–40	212.6	31.3	121.9	40.5
	40–48	159.3	125.0	82.4	.....
4.5	0–8	750	1.0	750	17.5
	8–16	744	14.3	644.8	43.2
	16–24	663.5	16.9	422.2	38.2
	24–32	574.0	23.9	293.2	73.3
	32–40	464.1	24.3	121.2*	.....
	40–48	373.8	53.8	.....	.....
	48–56	212.9	.....	.....	.....
9.0	0–8	1,500	4.1	1,500	12.4
	8–16	1,450.7	28.2	1,351.5	29.3
	16–24	1,123.2	20.3	1,034.9	85.2
	24–32	940.6	44.1	329.9*	.....
	32–40	608.5	71.6	.....	.....
	40–48	259.8*	.....	.....	.....
18.0	0–8	1,500	28.5	1,500	14.4
	8–16	1,158.1	28.5	1,327.7	54.7
	16–24	894.0	42.8	764.4	125.0*
	24–32	588.2	37.8	.....	.....
	32–40	410.5*	.....	.....	.....

* Extrapolation from partial data.

transfer technique. Therefore, the actual census figures do not directly give the information desired for this compilation but, instead, furnish age-specific death rates per number of individuals in any specified culture. These rates can be converted to a calculated number of the original population surviving (l_x of Table 4) by cumulative use of the following formula:

No. of original population surviving at beginning of interval − (Observed no. dying in interval) (No. of original population surviving) ÷ Total no. of animals in culture at beginning of interval.

The death rates (Table 4, 1,000 q_x) increase in general with increasing age, irrespective of density. During the earlier part of the life-span, death rates are highest at the higher densities. Other, more subtle, differences apparent in the unabridged life-tables are, of course, wiped out by this presentation, but their validity, on the basis of the original observations, is doubtful. It is particularly unfortunate that neonate and juvenile mortality could not be measured at various adult densities. Incomplete data suggest that at higher densities neonate mortality of *Daphnia* may be considerably higher than that of *Simocephalus*.

When one compares the effect of density on longevity of the species, it appears from Table 4 that a maximum life-span may be reached at intermediate densities. This is borne out by the median longevity of the two species at various densities (Table 5). In a descriptive sense the data are reminiscent of those reported by Pearl, Miner, and Parker (1927) for *Drosophila*. Table 5 can be used to compare the survival of the species. At rather low concentrations, *S.* has the longer median life-span; at higher densities the situation is reversed. The rather large difference in favor of *D.* diminishes, however, as extremely high densities are attained.

It is of some theoretical interest to compare more critically the shape of the survivorship curves as density increases. Figure 3 has been so constructed that differences in median longevity do not interfere with this comparison. The survivorship curves vary from the rectangular to the diagonal type (Pearl, 1940). The rectangular type may be interpreted to represent most closely the "physiological" longevity of the species (Bodenheimer, 1938). Thus, in the *Daphnia* cohort at a density of 0.3/cc, the great majority of individuals die during a short interval and may be presumed to have approached their maximum life-span for these conditions. At a density of 18/cc, on the other hand, the species exhibits a curve denoting "ecological" longevity: many animals die long before they have attained their maximum possible life-span.

TABLE 5

MEDIAN LONGEVITY OF SINGLE-SPECIES COHORTS AT VARIOUS DENSITIES

DENSITY (PER CC.)	MEDIAN LONGEVITY	
	Daphnia	*Simocephalus*
0.006.....	23.6	25.2
0.3.....	27.0	32.4
1.5.....	42.2	29.6
4.5.....	39.9	18.5
9.0.....	30.6	18.8
18.0.....	19.5	16.0

With these facts in mind, a more revealing examination of the greater average longevity at intermediate densities may be attempted. The phenomenon itself is quite general (Allee, 1931). In the present case there is some evidence that two separate effects are involved, particularly in the case of *Daphnia*. In this species a number of animals die at a relatively young age both above and below a density of 0.3/cc. It seems reasonable

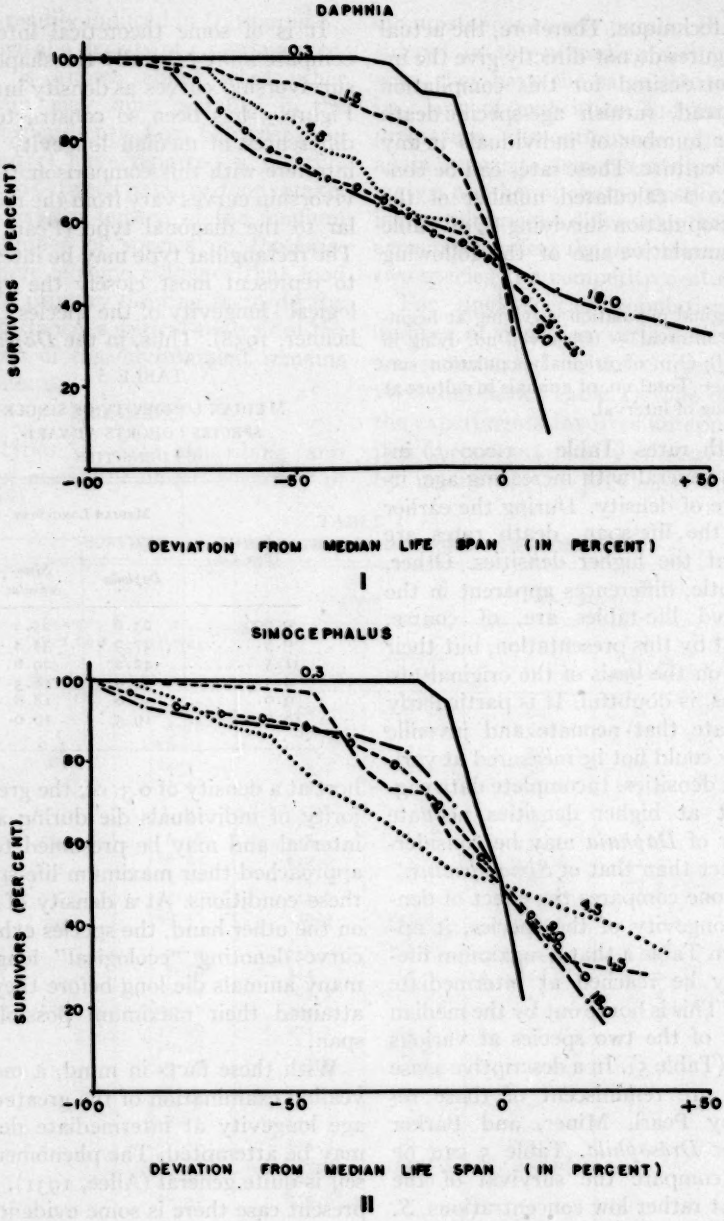

FIG. 3.—Survivorship of single-species cohorts at various densities. *I, Daphnia; II, Simocephalus*

that at the lower density this happens because the animals are unable to withstand or control some unfavorable factor in their environment. At densities above 0.3/cc, this explanation is less likely. MacArthur and Baillie (1929*b*) suggested that in *D. magna* survival is inversely proportional to the rate of living, which decreases within limits with increases in density. Fowler (1931) demonstrated a similar relationship between survivorship and metabolic rate of *D. longispina* exposed to certain toxic solutions. It seems probable that the increase in average survival of *D. pulicaria* at densities above c.3/cc. is another case of this sort. In the present experiments the only evidence comes from the observation that the length of instars increased with increases in density; the mechanism causing the presumed decrease in metabolic rate is not known.

The effects of differences in parental density on the survival of the offspring are as marked as was found to be the case for natality (Fig. 4). Median longevity at the higher parental density (9/cc, or 750 per half-sized bottle) is less than half that observed under conditions of lower parent density. It seems unlikely that the pronounced difference could be solely the result of the use of different culture vessels. As opposed to its effect on reproduction, parental crowding produces about the same relative decrease of longevity in both species.

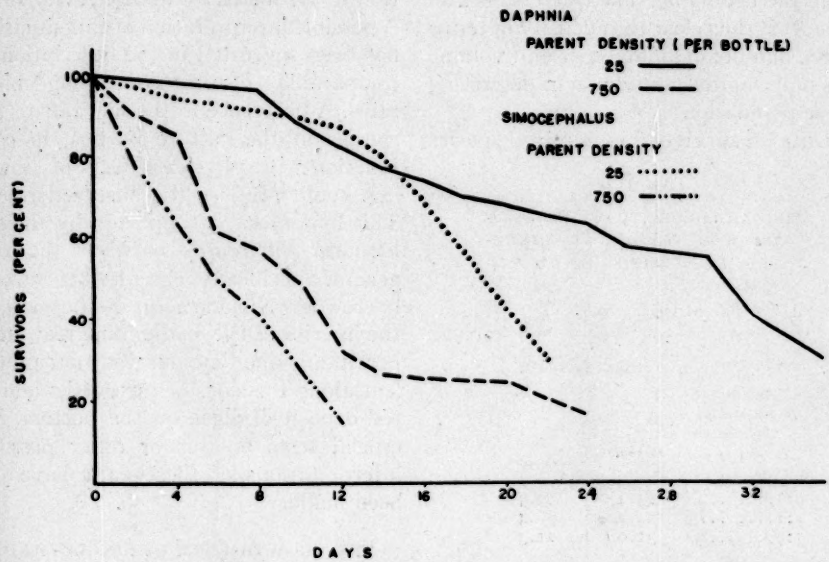

FIG. 4.—Effect of parent density on survival of offspring. Density of offspring, 9.0/cc

Sudden changes in density have about those effects on survival that might have been predicted. Suddenly increased density increases the death rate enormously (by a factor of 9 for *D.* and of 12 for *S.* in experiments paralleling those on natality). A decrease is accompanied by a smaller concomitant decrease in death rate. From the original data it appears that the effects of increased crowding manifest themselves at once to their full extent. When density is lowered, the full

change in survival is not observed until the sixth day following the change. It is pertinent to add here that animals raised at relatively low densities are larger and have shorter instars. The biomass of a number of animals raised under conditions of low density and then crowded more severely is therefore greater than that of animals crowded to that extent from the beginning. The converse is also true. It is thus clear that density in terms of the number of animals per unit volume has only limited usefulness in describing these populations.

Data on survivorship in mixed-species populations round out the observed effects of density on mortality. Appreciable differences as compared to single-species survival are detected only at the higher of the two total densities observed (6 animals/cc). At the extremely unbalanced ratio between the species (99:1 and 1:99), the numbers of the less frequent species are so small that estimates of their average longevity have little meaning. The relative performance of the species at the three remaining ratios can be assayed with more confidence (Table 6). Within the range of observed conditions, *S.* does not survive as long as *D.* The median life-span of *D.* is highest when its density is lowest, leading one to the conclusion that *S.* inhibits *D.* less than *D.* does itself. The longevity of *S.*, however, is greater when it or when *D.* is more abundant than when the ratio between the species is even. In other words, up to a certain point *D.* inhibits *S.* more than the latter does itself, but, beyond this, a further increase in proportion of *D.* increases the longevity of *S.* A possible interpretation of this situation has been suggested in the description of comparable results concerning birth rates. A difference in the habitats of the species in the culture bottles, in conjunction with physical effects of crowding, could produce the observed result. This hypothesis is supported by the behavioral differences between the two genera. Specifically, except when severely crowded, *Simocephalus* did not occupy the interior of the medium in great numbers at any one time but was more prevalent along the sides of the bottles and in the deposit of algae on the bottom. No crucial tests of this or other possible interpretations of these data have yet been made.

TABLE 6

MEDIAN LONGEVITY OF *Simocephalus* AND *Daphnia* AT MIXED-SPECIES DENSITIES OF 6.0 ANIMALS/CC

(Parent Density 0.6/Cc)

Ratio *Daphnia*:*Simocephalus*	Median Life-Span (Days)	
	Daphnia	*Simocephalus*
9:1.....	24.4	22.4
1:1.....	24.2	16.4
1:9.....	32.0	21.3

THE GROWTH-FORM OF SINGLE- AND MIXED-SPECIES POPULATIONS

The presentation of this, the best-documented set of data, is most readily accomplished by employing the following organization. First, the growth of single-species populations of both organisms will be analyzed and discussed in the light of their characteristics of mortality and natality. Certain similarities and differences in single-species population history will be pointed out. Finally, the mixed-species populations will be studied on the basis of all previous data in an attempt to explain the series of events culminating in the eventual extinction of *Simocephalus* in such cultures.

Figures 5 and 6 describe the temporal

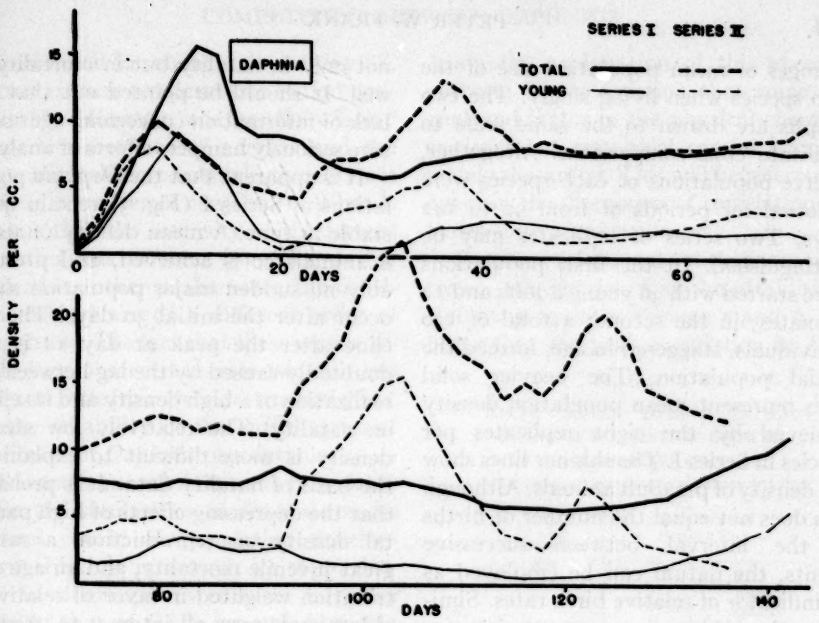

FIG. 5.—Single-species growth-form of *Daphnia*

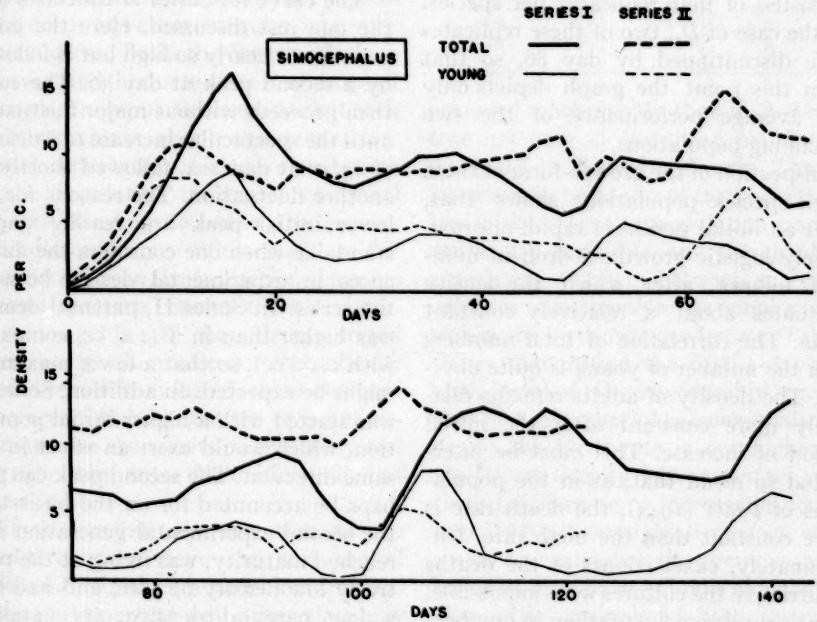

FIG. 6.—Single-species growth-form of *Simocephalus*

changes of mean population size of the two species when living singly. The two graphs are drawn to the same scale to facilitate cross-comparisons. Altogether, twelve populations of each species were followed for periods of from 90 to 142 days. Two series of replicates may be distinguished. In the first, populations were started with 36 young adults and 12 neonates; in the second, a total of 130 individuals, staggered in age, formed the initial population. The heavier solid lines represent mean population density achieved by the eight replicates per species in Series I. The thinner lines show the density of preadult animals. Although this does not equal the number of births in the interval between successive counts, the datum can be employed as an indicator of relative birth rates. Similarly, the broken lines present data for Series II, which, for the control cultures, consisted of four replicates per species. In the case of *D.*, two of these replicates were discontinued by day 86, so that from this point the graph depicts only the average performance of the two remaining populations.

Inspection of the growth-form of these single-species populations shows that, after an initial period of rapid, approximately logistic growth, a drop in numbers follows, after which the density fluctuates about a relatively constant mean. The correlation of total numbers with the number of young is quite obvious. The density of adults remains relatively more constant after the initial period of increase. This must be interpreted to mean that, as in the populations of Pratt (1943), the death rate is more constant than the birth rate. Unfortunately, exact counts of the deaths occurring in the cultures were impossible. Relatively severe fluctuations in numbers do occur at times, particularly in *S.*; here the almost complete population overturn in a short time is associated with changes not only in natality but in mortality as well. It should be pointed out that the lack of information concerning age structure seriously hampers efforts at analysis.

It is apparent that the *Daphnia* populations of Series I (Fig. 5) remain quite stable in time. A mean density of about 8 animals/cc is achieved, and presumably no sudden major population shifts occur after the initial 30 days. The decline after the peak at day 12 is undoubtedly caused by the lag between the realization of a high density and its effect on natality. The relatively low steady density is more difficult to explain on the basis of natality data. It is probable that the depressing effects of high parental density on reproduction, a rather great juvenile mortality, and an age distribution weighted in favor of relatively old animals may all interact to produce the end-result.

The curve for Series II contrasts with the one just discussed. Here the initial peak is not nearly so high but is followed by a second peak at day 36. The curve then proceeds without major fluctuation until the spectacular increase to a density of 25/cc at day 104, followed shortly by another fluctuation. The reasons for the lower initial peak are readily understandable when one considers the differences in experimental design between the series. In Series II, parental density was higher than in I (1.2/cc, compared with 0.15/cc), so that a lower maximum might be expected. In addition, Series II was started with a higher initial population, which would exert an effect in the same direction. The second peak can perhaps be accounted for on the basis that the second experimental generation had reached maturity, was living at the relatively low density of 5/cc, and had had a low parental density; its natality, therefore, should have been high. The other peaks defy adequate explanation at present. Opportunely, thermostatic

control failed for about a day at day 88, so that culture temperatures varied considerably. However, whether there is any connection between this event and the fluctuation observed shortly thereafter is entirely a matter of conjecture. What is clear is that the intensity of the factors operating was different from that acting to produce other peaks, since the densities reached are of a different order of magnitude. The fact that these erratic results were observed in only two cultures detracts little from their destructive effect on the reliability of the entire series of replicates of *Daphnia* populations.

Simocephalus exhibits some previously indicated similarities to *Daphnia* in its general course of population development (Fig. 6); moreover, its mean density in single-species cultures is rather close to that of *D.* In general, however, population density fluctuates to a greater degree. The alternate peaks and depressions occur at rather irregular intervals. Nevertheless, both the length of these intervals and the fluctuation in numbers of young in the population suggest that each cycle represents about one generation. Stated differently, the age distribution of the population undergoes continual changes, tending to make the generations discontinuous. While there are differences between the two series, the same general pattern emerges from both. Hutchinson (1948) has demonstrated that such fluctuations theoretically should result from lag effects of the type that occurs in both species. It is not apparent from the results on mortality and natality why such fluctuations do not occur in *D.*

If we confine our attention to the period between days 100 and 102 of Series I *S.* cultures, a phenomenon without counterpart at any other time may be observed. The population change indicates that heavy mortality is accompanied by a high reproductive rate. Again the explanation of this event is obscure. The suggestion that some unanalyzed factor in the physical-chemical environment may exist cannot be completely discarded. This will be referred to again in the discussion of correlation between individual replicates.

Certain additional differences between the species may be established. Referring again to Figures 5 and 6, one notes that the peak population in *D.* is achieved more quickly than in *S.* This is true not only of the mean populations but of all individual replicates. The difference may be analyzed further. During the initial stages of population growth, deaths are negligible, and the change in population numbers between successive censuses can therefore be employed to obtain the birth rate per female. Table 7 shows that during the first 4 days the birth rate of *D.* considerably exceeds that of *S.* Another phenomenon also appears; the birth rate is not highest at the lowest density but at a stage when there has been some increase in population. This optimum density effect, which seems to contradict the previous natality data, may have a simple explanation. The initial animals in both series are preponderantly relatively young; most of them have not yet reached their reproductive peak. As a result, their reproduc-

TABLE 7

BIRTH RATES DURING INITIAL PHASE OF POPULATION GROWTH OF SINGLE-SPECIES CULTURES

Population Age (Days)	Natality per Female Day	
	Daphnia	*Simocephalus*
2.....	3.0	1.4
4.....	3.7	1.7
6.....	2.3	2.5
8.....	1.2	1.2
10.....	0.6	0.6

tivity as a group increases for a short period. The change in density presumably does not influence this increase at first, because of the demonstrated lag between changes in density and changes in reproductive rate. Furthermore, at about the same density, *D.* produces a larger number of young early in its life-span than does *S.* It is, therefore, reasonable to assume that the difference in the rate of increase of the populations during the first few days may result merely from the way in which the experiments were initiated. With the attainment of higher densities, the difference could become accentuated as a result of the greater effect of density on the natality of *S.*

TABLE 8

VARIABILITY BETWEEN REPLICATES OF SINGLE-SPECIES POPULATIONS OF *Daphnia*

Age (Days)	Series I						Series I and II					
	Adults			Young			Adults			Young		
	Mean	*N*	C.V.	Mean	*N*	C.V.	Mean	*N*	C.V.	Mean	*N*	C.V.
10.....	593	8	10.2	1,181	8	5.4	498	12	31.0	1,226	12	9.4
20.....	1,175	8	6.3	267	8	18.6	987	12	29.5	241	12	24.5
30.....	920	8	8.7	108	8	71.1	803	12	23.9	277	12	113.1
40.....	761	8	13.3	377	8	47.7	862	12	22.5	563	12	65.7
50.....	947	8	10.8	367	8	47.4	1,020	12	14.7	309	12	53.1
60.....	944	8	16.6	339	8	22.3	949	12	15.8	342	12	19.0
70.....	1,016	8	11.7	308	8	38.4	972	12	13.2	398	12	47.4
80.....	808	8	17.8	343	8	54.2	911	11	24.1	458	11	54.4
90.....	813	8	13.5	398	8	49.5	916	10	27.2	419	10	43.4
100.....	994	8	12.6	243	8	65.0	1,041	10	16.9	536	10	121.7
110.....	961	8	13.3	171	8	45.2	1,074	10	24.5	454	10	132.4
120.....	856	8	6.3	85	8	48.9	1,067	10	42.2	150	10	114.9

So far, an attempt has been made to establish basic patterns of population growth-form of the single-species populations. This depends, however, not so much on mean population size as on the variability and correlation between individual replicates. Data on the variation observed are afforded by Tables 8 and 9, which present actual mean populations for both species, the number of replications, and the coefficient of variability for Series I alone and combined with Series II. Data are presented for arbitrary 10-day intervals. Within a series the variability is no higher than what one might expect in data of this sort with so few replicates. When the two series are combined, variability, of course, at times becomes great, partly because the series are out of phase. In general, it may be noted that the variation in the number of young is greater than that in the number of adults.

The results suggest that the mean values do have some validity; they do not, however, give a complete picture of the correlation in population change that exists, particularly between individual cultures of one series. The degree to which populations fluctuate in synchrony is astonishing, and once again raises the issue of uncontrolled environmental causation. Only the most extreme case will be presented (Fig. 7). The eight replicate *S.* cultures of Series I are here graphed from day 80 to day 110; separation of the curves along the vertical axis

was necessary to make the graph legible. It is quite striking that, with few exceptions, population changes occur simultaneously in all replicates. This case is not isolated but only one of a number of such events in *D.* as well as in *S.* In order to clarify the sources of such correlation, some additional information regarding experimental design is necessary. There are two groups of four cultures each; the members of a group are bracketed together at the right of the figure. All replicates in one group were counted on the same days and received the same medium. Those of the other group were counted on alternate days and were fed different algal cultures. In view of this fact and the constant light and temperature that were maintained, it is difficult to see what factor in the purely external environment can be responsible for such synchrony.

Sufficient analysis of the single-species record has now been presented to make certain critical evaluations possible. One can state categorically that neither species was ever threatened with extinction during its observed population history. A number of differences between the growth-forms of the two species under identical conditions exist; many of these cannot be fully explained. The mean density of the species when grown singly is very similar, and certainly no a priori judgment of the behavior of the two when living together can be made by this criterion alone.

TABLE 9

VARIABILITY BETWEEN REPLICATES OF SINGLE-SPECIES POPULATIONS OF *Simocephalus*

Age (Days)	Series I						Series I and II					
	Adults			Young			Adults			Young		
	Mean	*N*	C.V.	Mean	*N*	C.V.	Mean	*N*	C.V.	Mean	*N*	C.V.
10.....	344	8	9.4	634	8	10.1	313	12	17.6	831	12	37.8
20.....	1,091	8	3.3	1,032	8	4.6	929	12	27.8	935	12	29.4
30.....	896	8	5.2	394	8	70.5	828	12	15.3	519	12	61.5
40.....	1,004	8	16.9	668	8	41.1	956	12	17.3	666	12	26.3
50.....	801	8	5.8	139	8	21.3	961	12	25.4	287	12	85.2
60.....	892	8	5.9	667	8	26.4	984	12	14.6	513	12	52.2
70.....	920	8	13.8	314	8	52.0	1,032	12	21.5	521	12	60.5
80.....	714	8	8.3	165	8	65.9	1,156	12	35.5	217	12	68.5
90.....	691	8	17.8	623	8	27.0	911	12	37.2	629	12	22.1
100.....	700	8	6.4	89	8	54.1	853	12	31.0	222	12	104.8
110.....	586	8	27.4	1,313	8	17.7	868	12	49.1	1,076	12	38.1
120.....	1,837	8	15.0	238	8	30.1	1,744	12	26.4	275	12	40.4

A visual description of what happens when both species are cultured together is given in Figure 8. Series I (consisting of eight replicates started with 48 animals per secies) and Series II (with six replicates of initial density of 24 per species) could be combined, since they revealed no major differences. The vertical scale for *S.* has been exaggerated three times over that of *D.* Both species exhibit an initial increase in population. Comparison with the single-species records, which may be used as controls, at once reveals that the density of *S.* is markedly limited by the mixed-species environment. During the first 30 days this is also true for *D.*, but to a much less ex-

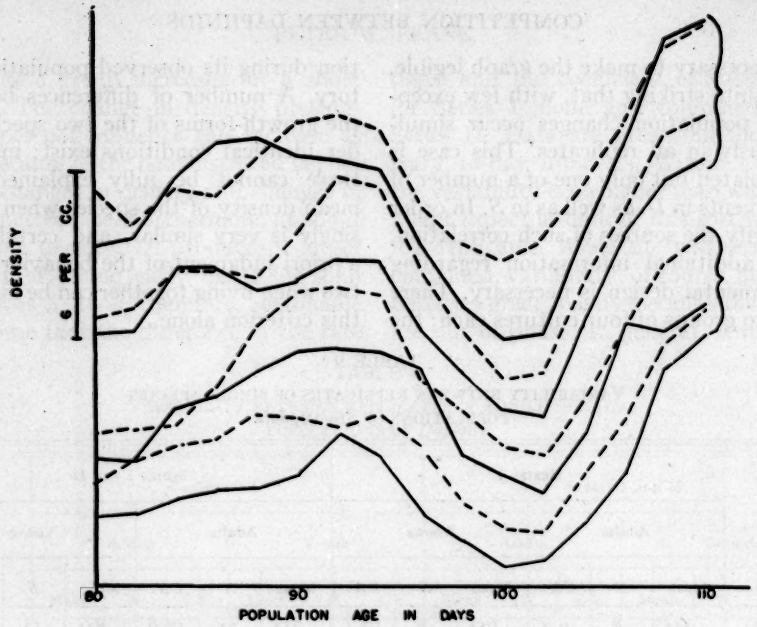

FIG. 7.—Correlation between eight single-species *Simocephalus* populations

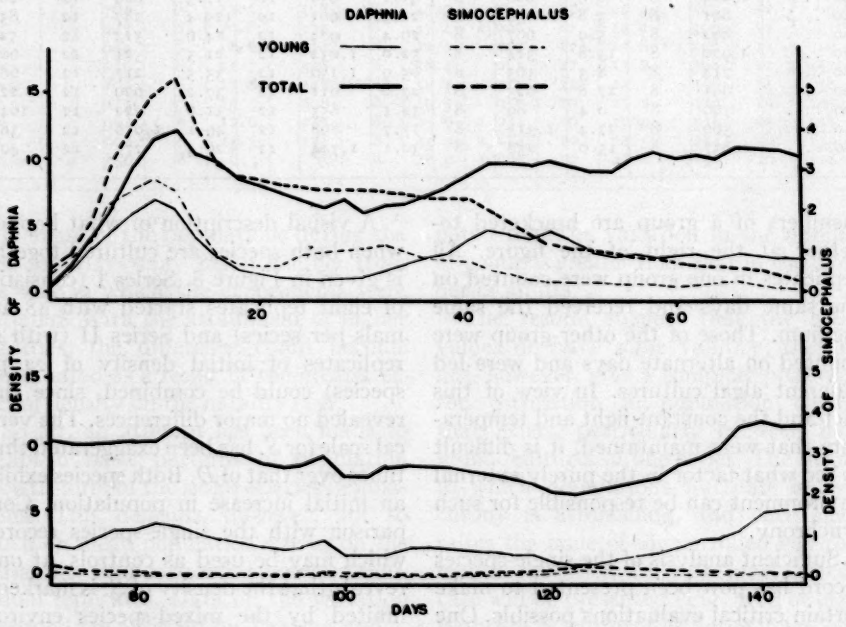

FIG. 8.—Growth-form of *Daphnia* and *Simocephalus* in the competitive mixed-species situation

tent. For example, at day 10 the total mean difference in actual numbers between single- and mixed-species populations of *D.* is 214 ± 68.1, whereas the respective values for *S.* are 469 ± 94.8. At later stages in population development, the mixed-species *D.* populations become statistically indistinguishable from the controls, whereas in *S.* the difference continues to increase. The range of variance of these mixed-species populations closely approximates that of the control replicates, except that the coefficients of variability for *S.* in the competition series rise to much higher levels after the first 40 days. The decline in numbers of this species therefore proceeds at quite diverse rates in different replicates. Eventually, the decline ended in extinction in all mixed-species cultures observed. Little meaning can be attached to mean time of extinction, however, since the range is very great. A parallel situation has been described in *Tribolium* (Park, 1948); a few individuals of the species that ultimately becomes extinct may persist for some time.

The first case of extinction of *S.* occurred at a population age of 78 days. Five additional populations died between days 80 and 100, five more between days 100 and 150, one became extinct between days 150 and 200, and the remaining two replicates between days 200 and 222. Even these data give little indication of the population changes during the often extended period when the density of *S.* was very low. Let us take a specific example, that of replicate H6. By day 76, *S.* had declined to the extent that the population consisted of one adult. Between day 88 and day 90, this animal produced two young; it had another brood of four on day 94. Subsequently, the numbers fluctuated at a low level for some time. The maximum population attained was 44 (0.27/cc) at day 132. Minor fluctuations occurred, until on day 216 only one adult, which died 6 days later, remained.

There may be some reality in dividing the population growth-form of *S.* in these mixed-species populations into three separate phases; the procedure

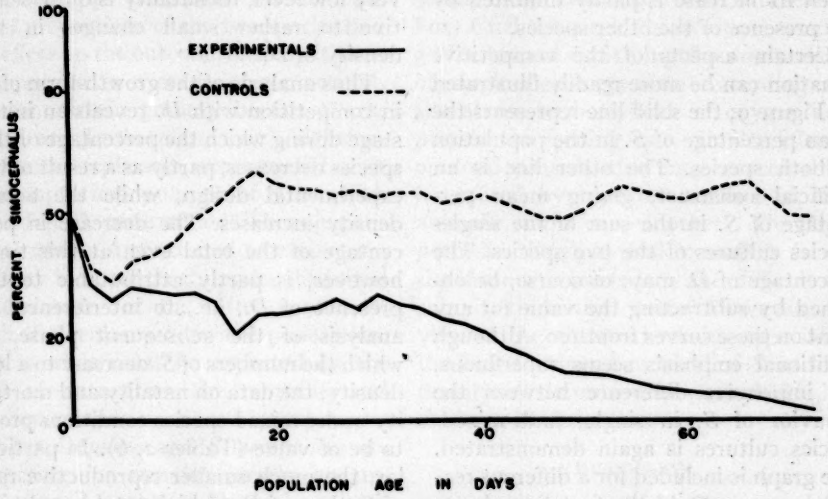

FIG. 9.—Relative performance of *Simocephalus* in single- and mixed-species environments

certainly is justified for purposes of description. An initial period of increase is followed by a more or less rapid decline to a very low density. Finally, there is a variable period of time prior to extinction, during which the population remains at a low level. *Daphnia* in the same cultures shows population development not very different from the control replicates except for the initial period, when its increase is partly inhibited by the presence of the other species.

Certain aspects of the competitive situation can be more readily illustrated by Figure 9; the solid line represents the mean percentage of *S.* in the population of both species. The other line is an artificial construct, giving mean percentage of *S.* in the sum of the single-species cultures of the two species. The percentage of *D.* may, of course, be obtained by subtracting the value for any point on these curves from 100. Although additional emphasis seems superfluous, the impressive difference between the behavior of *S.* in single- and mixed-species cultures is again demonstrated. The graph is included for a different reason, however. Only the first two phases of *S.* population history are presented. It is apparent that during the initial phase the drop in the percentage of this species is accounted for at least in part by the initial conditions; the similar decline in the control populations has been adequately discussed previously. However, although *S.* in the control populations increases its percentage of the total quickly, such recovery is not accomplished under mixed-species conditions. Until about day 32, the percentage of *S.* does not decrease significantly below that at day 4. This is correlated with the decreased numbers of *D.* after the initial peak. While it is not apparent from Figure 8, in approximately half the replicates *S.*, in addition, shows slight increases during this period. As the numbers of *D.* once again increase, the percentage of *S.* and its actual numbers drop rather quickly and consistently. Similar inverse correlations between the densities of the two species could be observed also around days 100 and 120. Because of the small numbers involved, this observation is not clear from Figure 8. Apparently, even after *S.* has reached a very low level, its natality is quite sensitive to rather small changes in the density of *D.*

Thus analysis of the growth-form of *S.* in competition with *D.* reveals an initial stage during which the percentage of this species decreases, partly as a result of the experimental design, while the actual density increases. The decrease in percentage of the total even at this time, however, is partly attributable to the presence of *D.*, i.e., to interference. In analysis of the subsequent phase, in which the numbers of *S.* decrease to a low density, the data on natality and mortality under mixed-species conditions prove to be of value (Tables 2, 6). In particular, the much smaller reproductive rate of *S.* than of *D.* at high total population density and a fairly evenly balanced ratio between the species can be expected to contribute to the observed decline. As was indicated in the discussion of these data, when the proportion of *S.* becomes quite low, its relative performance with respect to natality and survivorship improves considerably. This presumably accounts for the extended period during which *S.* survives at a very low density. It is quite likely that extinction of the species within a replicate occurs only as a result of the unstable age distribution which such a small population must possess. At any one time, all the surviving individuals may be close to the same age; if a particularly high density of *D.* happens to coincide with a period in

which all the *S.* happen to be old, extinction is very probable. In other words, if the complete environment could be enlarged by a factor of, say, 10, thus increasing the numbers of both species proportionately, it seems probable that extinction of *S.* would not occur. Instead, the latter species might maintain itself at low density in the presence of a high density of *D.*

In order to determine whether the initial proportions of the two species had any effect on the outcome of competition, as happened in a study of competition between certain granary beetles (Park, Gregg, and Lutherman, 1941), eight experimental replicates were started with an asymptotic population of *S.* to which two adult *D.* were added. These experiments were not continued to extinction, but they indicate that, despite the initial disadvantage for the latter species, it gradually replaces *S.* in the population. This is what must be expected on the basis of data from the mortality and natality assays.

What *Daphnia* and *Simocephalus* are competing for remains largely an uninvestigated problem. It resolves itself mainly into the question of what factors are able to limit single-species populations. Four general causes for such limitation of numbers may be cited: a quantitatively or qualitatively insufficient food supply, chemical conditioning by volatile or nonvolatile factors, oxygen lack, and physical effects of crowding. The last possibility implies that mortality and natality are affected, although not necessarily directly, by some sort of sensory stimulation which is a function of the density. Pratt (1943) demonstrated that for *D. magna*, under somewhat similar circumstances, chemical conditioning by nonvolatile factors played no significant role. Slobodkin (1951) found food to be the sole limiting factor for his populations of *D. obtusa* and suggested that the same was true with the *D. magna* of Pratt. In the present case we are dealing with somewhat higher densities; preliminary experiments indicate that neither food nor chemical conditioning by nonvolatile substances is of great significance in limiting population density. Oxygen lack may prove to be involved. If this is shown to be true, the recent studies of Fox, Gilchrist, and Phear (1951) would go far toward explaining the mechanism of single-species growth. According to the above authors, lack of oxygen depresses the feeding rate. Similar effects on food consumption may conceivably be caused by repeated contact of the animals with one another. The hypothesis that physical crowding is of significance is hazardous and difficult to submit to critical tests. However, in view of the observed results, especially the long-continued survival of *S.* at low density in the mixed-species cultures, it deserves investigation.

DISCUSSION

Competition undoubtedly causes the extinction of *S. vetulus* when this species lives with *D. pulicaria* under certain experimental conditions. It also seems clear that extinction results in part from intrinsic differences between the mortality and the natality of the two forms and in part from the greater power of interference displayed by *D.* The mutual inhibition of the species varies with density in a rather complex manner. An attempt has been made to interpret this in terms of the different habitats of *D.* and *S.* in the culture bottles.

Crombie (1945) has reviewed the mathematical models of interspecies competition and has verbalized their implications. Of the four possible consequences of interspecific competition, two

are of interest here: (1) if each of two species inhibits its own potential increase more than that of the other, both continue to exist together; (2) if the second species inhibits the increase of the first less than its own, while the first inhibits that of the second more than its own, the first species drives out the second, irrespective of the initial numbers of the two species. The mathematical models from which these conclusions derive assume that competition is a linear function of the densities of the two species involved. Only a beginning has been made in the mathematical investigation of the probably more common non-linear relationships (Hutchinson, 1947).

Nevertheless, if we compare these theoretical statements with the present results, it is apparent that no great divergence exists. *Daphnia* is inhibited more by itself than by *S.* The latter species is inhibited about equally by itself and by *D.*, until its proportion of the total density becomes only a small fraction of the total, when *D.* is shown to inhibit it to a much smaller extent. The observed competitive situation is thus intermediate to the conditions demanded by the two theoretical statements, and the observed end-result is readily reconciled with the theoretical outcome. This empirical evidence, therefore, adds a little more weight to the theory of interspecies competition; at the same time, it points out certain discrepancies between the simplified mathematical models and a simplified biotic situation. A general statement of this theory has been presented in the introduction to this study. Extinction of all but one species occurs whenever there is sufficient similarity in the environments and in the needs and habits of competing forms. The laboratory environment of the two species has been so designed that competition between them is intense. Nevertheless, there is some differentiation into rudimentary niches, the interior of the medium, and the sides and bottom of the container. Since their needs and habits are not identical, *D.* and *S.* presumably are able to exploit these niches differentially. As a result, competition is diminished to the point where *S.* is almost able to persist.

Applications of the present results to the problem of distribution and density of these species in nature require considerable care. The present study was not primarily intended to furnish information of this sort. There are many major differences between the experimental situation and that obtaining in a lake or pond. Presumably, one of the results of these differences is that the densities of the species in the field fall far below those attained in the laboratory. Thus Eddy (1934) gives peak densities of 0.054/cc for *S. vetulus* in a temporary pond; of 0.022/cc for *D. pulex*, and of 0.28/cc for *D. longispina* in certain permanent ponds. Ward (1940) estimates the density of maximum total Cladocera in a number of small ponds at between 1 and 2/cc, and Pennak (1946) gives a figure of 0.435 for Gaynor Lake, a larger body of water. It is probable, however, that densities of the order of magnitude observed in the present investigation may occur temporarily in small ponds of high organic content. In view of the natural densities reported, however, it is likely that, if competition between such species occurs, it is largely for food and in terms of intrinsic differences in natality and mortality rather than of interference. Competition would tend to be reduced also because of the greater possibilities of differences in dispersion.

The occurrence of a sexual process accompanied by delayed hatching of the eggs tends to make extinction as a result of competition an event of short duration for these species. Conditions in

small ponds are so variable that a species which had been locally extinct for a time might, upon hatching of sexual eggs, find conditions for it nearly optimal. Dispersal of the sexual eggs seems to occur with considerable ease. Extinction because of competition thus is probably of little significance in the distribution of many daphnids. This is borne out by their wide, although sometimes erratic, geographic distribution. The relative numbers of different species in the same body of water are presumably influenced by competition, but only in conjunction with a whole complex of other factors that can limit population growth.

Since more severe competition may be expected between animals with more similar needs, intraspecific competition between different genotypes may be of considerable significance in species of the reproductive pattern of Cladocera. During the often prolonged period of exclusively asexual reproduction, such competition within a species is similar to interspecies competition in effect and may lead to the extinction of all but one genotype, at least in theory. With the recurrence of sexual reproduction, genetic variability would, of course, be partly restored. However, under such conditions, competition may be a particularly potent factor in natural selection.

SUMMARY

1. The growth-form of single- and of mixed-species populations of *D. pulicaria* and *S. vetulus* has been observed through several generations under relatively constant laboratory conditions.

2. In single-species populations the average steady density of the two species was about the same, and, although differences in relative stability of numbers seem to exist, neither species ever approached extinction.

3. In the mixed-species populations, *S.* invariably died out, while *D.* assumed the behavior characteristic of single-species cultures even before complete elimination of the other species.

4. Birth and death rates of single- and mixed-species cohorts have been shown to be markedly affected by a number of conditions related to density.

5. Analysis of the population histories in terms of the latter data, despite discrepancies, reveals substantial agreement.

6. Although there has been no conclusive study of the environmental resources for which the species compete, several possibilities are discussed.

7. The present results can be integrated into the general picture of interspecific competition theory.

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